Convergent Evolution in Two Bacterial Replicative Helicase Loaders

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Abstract (120 words)

Dedicated loader proteins play essential roles in bacterial DNA replication by opening ring-shaped DnaB-family helicases and chaperoning ssDNA into a central motor chamber as a prelude to DNA unwinding. Although unrelated in sequence, the *E. coli* DnaC and bacteriophage λ P loaders feature a similar overall architecture: a globular domain linked to an extended lasso/grappling hook element, located at their amino and carboxy termini, respectively. Both loaders remodel a closed DnaB ring into nearly identical right-handed open conformations. The sole element shared by the loaders is a single alpha helix, which binds to the same site on the helicase. Physical features of the loaders establish that DnaC and λ P evolved independently to converge, through molecular mimicry, on a common helicase opening mechanism.

Keywords

DnaB, DnaC, λ P, DciA, DNA Replication, helicase loading, convergent evolution, molecular mimicry.

Introduction (~3100 words)

Specialized Factors Load the DnaB Bacterial Replicative Helicase onto the Replication Origin

The regulated loading of ring-shaped hexameric helicases onto chromosomal origins is an essential feature of DNA replication in all cellular domains of life [1–3]. Helicase deposition requires specialized factors known as helicase loaders, which operate during the initiation of DNA replication [4–8]. In bacteria, several helicase loaders have been studied, including *Escherichia coli* (*E. coli)* DnaC [4], bacteriophage λ P, *Bacillus subtilis* (*B. subtilis*) DnaI [5], and DciA/DopE, a recently described class of proteins which appear in bacteria that lack orthologs of DnaC or DnaI [6,7,9]. Helicase loading is also critical to assembly of eukaryal and archaeal **replisomes** [1,2,8].

The assembly of the bacterial replicative helicase (**Figure 1**), which is known as DnaB in most bacteria or DnaC in *B. subtilis* (henceforth called DnaB), onto origin DNA occurs during the initiation phase of DNA replication [8,10–14]. Our view of replication initiation in bacteria is informed by studies with primary and secondary chromosomes of bacteria, plasmids, and phages [15–27], and have implied the involvement of four classes of factors (**Table 1**): 1) a DNA sequence called a replication origin, where DNA synthesis will begin [13,20,28,29], 2) a replication initiator protein (*E. coli*: DnaA [10,12,30–32], *V. cholerae*: DnaA, RctB [18,21,23,25,33–37], plasmids: RepE, Pi, TrfA [38–40], phage lambda (λ): O [41–49]), 3) a DnaB-family replicative helicase [4,12–14,50], and, finally, 4) a helicase loader (*E. coli:* DnaC [4,10,16,51–55], phage λ: P [43,45–48,56–59], *V. cholerae*: DciA [6,9]). The multi-step process for initiating DNA replication begins with the recognition and binding of multiple copies of the initiator protein to dsDNA sites at the

replication origin; once bound, initiator proteins associate into a complex protein-DNA ensemble [4,10,12,19,31,60,61]. One of the outputs of the initiation phase of DNA replication is the melting of an A-T rich segment of the replication origin termed the DNA unwinding element (DUE) by the DnaA or the λ O initiator proteins [13,30,31], an event that provides single DNA strands (ssDNA) as substrates for DnaB loading. DnaB hexamers assemble into two-tiered rings formed by the amino (NTD) and carboxy-terminal domains (CTD) of the helicase; a so-termed 'linker helix' element (LH) connects these domains and packs against another alpha helix, termed the docking helix (DH), of a neighboring DnaB subunit to give rise to a **domain-swapped oligomer** [62–64] (**Figure 2**). The two DnaB tiers circumscribe an internal chamber into which one of the ssDNA strands from the replication origin will be loaded. One layer is formed out of six NTDs, which assemble into two different 'trimer-of-dimers' configurations that display pseudo-three-fold symmetry. These arrangements arise from alternative packing orientations for NTD dimers, which create several types of subunit interfaces of likely varying stability [62,65]. The CTD tier assembles out of six C-terminal domains (CTD) of DnaB, each of which harbors a **RecA-like ATPase domain**. In contrast to the NTD, the CTD layer exhibits a pseudo-six-fold arrangement, with a single type of interface.

Helicase loading onto ssDNA can be conceptually divided into four stages (**Figure 1**). During assembly, DnaB transitions between three conformations: closed planar, open right-handed spiral, and closed right-handed spiral [62–68]. In addition, the NTD and CTD layers of each of these conformers are found in one of two arrangements: dilated or constricted (**Figures 2A-2B**); these conformers differ on inter-protomer contacts. The isolated hexameric helicase, in both dilated and constricted closed-planar configurations,

74 populates Stage I. Formation of the helicase•helicase loader complex leads to Stage II,
75 while engagement of the helicase•loader complex with origin-derived ssDNA•initiator
76 (DnaA or λ O) populates Stage III. The ATPase and ssDNA translocation activities of the
77 helicase are suppressed during these latter two stages [56,58,69–71], with the
78 DnaA/replication origin complex also playing a role in loading or positioning the
79 helicase/loader complex using direct contacts between DnaA and DnaB [69–71] (the
80 involvement of contacts between other bacterial replication initiators and DnaB remains
81 to be clarified). Recently, cryogenic electron microscopy (cryo-EM) analyses of two
82 distinct bacterial helicase•loader complexes (*E. coli* DnaB•DnaC and *E. coli* DnaB•λP,
83 **Figure 1: Stages II-III, and Figure 3**) have shown that the helicase adopts an open right-
84 handed spiral configuration, promoted, and stabilized by interactions with the helicase
85 loaders [65,68]. The transition to Stage IV is accompanied by eviction of the loader and
86 initiator from the complex on DNA, which relieves inhibition of DnaB's activities. Notably,
87 in Stage III, the configuration of the DnaB-bound loader is nearly identical to the closed
88 spiral form seen in the loader-free helicase of Stage IV [65,67].

89 Helicase loading in bacteria occurs by one of at least two reported mechanisms
90 [72], termed: a) ring breaking, where DnaB hexamers are physically opened [73], and b)
91 ring making, in which hexamers are assembled [5]. It is now clear that both *E. coli* DnaC
92 and phage λ P are ring breakers: each loader binds to and delivers a pre-formed helicase
93 hexamer to its cognate origin [4,43,45–47,56,58,74]. DnaC and λ P are similar in that
94 both are essential for their respective organisms and both bind to ssDNA [56,58,75–77].
95 λ P also can displace DnaC from *E. coli* DnaB, implying that their respective binding sites
96 overlap [58,74]. These congruencies might imply a common ancestry; however, DnaC

and λP are unrelated in sequence and enzymatic function (e.g., DnaC is a known ATPase [10,53,75–77], whereas λ P is not [78]). Moreover, although each loading system requires ejection of the loader from the DNA complex for DnaB to transition to the translocation-competent form, eviction occurs by distinct mechanisms. For DnaC, nucleotide dynamics in its **AAA+ ATPase domain**, along with RNA synthesis by the DnaG primase, are significant features in eviction [10,52,53,76,77,79]. By contrast, removal of λ P requires the host DnaK/DnaJ/GrpE chaperone machinery [31–34]. Helicase loading is also a feature during restart of DNA replication after it has prematurely been halted in response to DNA damage and involves a distinct set of proteins (PriA, PriB, PriC, and DnaT); the reader is referred to the literature for a more complete treatment of helicase loading during replication restart [80–82].

Here, we compare recent structures of two bacterial helicase loaders: *E. coli* DnaC [65] and phage λ P [68] bound to the same *E. coli* DnaB helicase (**Figure 3**). This comparison provides an opportunity to understand the mechanisms of the two loaders and to extract central principles associated with DnaB opening and loading onto ssDNA. A recent crystal structure of the interaction domains of DnaB and DnaC [83] comports with the EM analyses of the complete complexes. A new study of DciA•DnaB interactions also points to some conserved elements of helicase opening in this system as well [9]. Further study will be required to establish the mechanistic relationships, if any, between the DciA and *B. subtilis* DnaI/DnaB/DnaD loaders [5,84,85] and the better understood *E. coli* DnaC [65] and λ P [68] systems.

Though unrelated by sequence and fold, *E. coli* DnaC and λ P exhibit analogous global architectures

Inspection of the *E. coli* DnaB•DnaC (BC) and the *E. coli* DnaB•phage λ P (BP) complexes shows that the two loaders engage the carboxy-terminal (CTD) ATPase surface of DnaB to form a three-layered ensemble [65,68,83]. One layer corresponds to an oligomeric form of the helicase loader (hexameric for DnaC and pentameric for λ P), while the second and third correspond to the NTD and CTD tiers of DnaB (**Figures 3A-B**). Although six copies of DnaC are present in the *E. coli* BC complex and five λ P protomers are found in the BP assembly, the stoichiometries seen in the cryo-EM structures do not necessarily preclude the possibility that complexes with fewer copies of the loader might be active in supporting helicase loading. Significantly, each loader adopts a distinct and unrelated structure, yet the monomers of both exhibit a similar overall architecture: a globular domain fused to an extended segment that forms a lasso/grappling hook element (**Figures 3C-D**).

The globular domain of DnaC consists of an AAA+ ATPase module that is fused to a ~75 residue N-terminal segment (**Figure 3C**)[65,83]. The amino-terminal segment of DnaC consists of a long α-helix that extends along the CTD of a DnaB protomer and initiates from a helix-loop-helix element that packs against the LH linker helix from one DnaB subunit and the DH docking helix from another. Notably, the N-terminal segment provides the only contacts between DnaC and the DnaB helicase. The six copies of the globular DnaC AAA+ domain assemble into an open spiral like that seen in related ATPases such as DnaA and archaeal/eukaryal MCM helicases [86–92]. In the absence of ssDNA, five of the six nucleotide-binding sites in DnaC are populated with an ATP

analog (ADP•BeF_3), whereas the sixth (which sits at the gap in the DnaC spiral) engages ADP, likely because its catalytic center lacks important functional contacts from a neighboring protomer. Rationalizing this arrangement of nucleotides is the prior finding that the ATP form of DnaC suppresses DnaB's helicase activity and as it stabilizes the ssDNA complex [52].

For the λ P loader, only the C-terminal ~125 residues were resolved in EM density maps [68]. This region consists of an α-helical globular domain fused to an extended segment of ~45 residues (**Figure 3D**). The λ P extension forms a sub-structure analogous to, but distinct from, that seen in DnaC [65,83], terminating in a single α-helix that packs against the LH and DH elements of two adjacent subunits of DnaB. Both the globular domain and the grappling hook/lasso segment of λ P contact two consecutive subunits of the DnaB hexamer. These interactions are repeated in the five copies of λ P in the BP complex to create an open helical arrangement of loaders; the breached interface of the DnaB hexamer precludes binding of a sixth copy of λ P. The low resolution (4.1 Å) of the BP EM maps in the region of the loader limits analysis of interfaces between λ P protomers; nevertheless, an extensive interface between the five λ P protomers does not appear to form.

E. coli DnaC and phage λP reconfigure DnaB into an open right-handed spiral

Despite their evolutionarily distinct structures and contacts with DnaB, both DnaC and λ P reconfigure the helicase into highly similar, open-spiral configurations (root mean square deviation (RMSD) of ~2.2 Å, calculated from 2611 Cα positions that span the

DnaB hexamer) [65,68]. The DnaB NTDs also both adopt a constricted configuration, albeit with a spiral (as opposed to planar) shape that bears a split between one of the subunit interfaces. The similarity between DnaB in the two loader complexes is also evident from the average helical pitch and twist values of the open spirals in both the NTD (~2.8 Å and 60.0° for BC *vs*. ~2.6 Å and 59.9° for BP) and CTD layers (~19.3 Å and ~55.3° for BC *vs*. ~16 Å and ~56° for BP). Changes induced by each loader rupture one of the DnaB subunit interfaces at both the NTD and CTD layers to create openings (15-20 Å) of sufficient size to allow ssDNA access to the internal chamber.

Changes to the helical pitch and twist of the DnaB hexamer within each loader complex combine to alter the configuration of the ssDNA-binding site in the helicase. Superposition of DnaB from each loader complex reveals significant changes in the position of DNA binding residues in comparison to that when DnaB is bound to ssDNA (**Figure 4)** [65]. When bound to ssDNA and the loader, the CTD of each DnaB protomer projects three residues (*E. coli*: R403, E404, G406) on a DNA binding loop into the helicase pore to contact ssDNA. In the loader-only complexes, reconfiguration of the CTD layer shifts the positions of the alpha-carbons of these residues by ~10-30 Å (in BC) or ~5-20 Å (in BP).

The disposition and nucleotide occupancy of the six RecA-type ATPase sites in DnaB are also altered in the complexes with DnaC and λ P [65,68]. ATPase activity by DnaB relies on ‘composite’ nucleotide binding sites, wherein residues from two adjacent subunits contribute to a single catalytic center [63,64,67,93]. In both helicase•loader complexes, five of the six ATPase sites in DnaB are occupied by ADP, while the sixth, which sits at the breach in the CTD ring, is vacant; when bound to just ssDNA, this

187 constellation of sites are filled with a nucleoside triphosphate analog (ADP•BeF_3) instead.
188 The alterations in CTD orientation appear to have remodeled the five ADP-filled sites of
189 the loader-bound helicase into non-optimal catalytic configurations as well, although the
190 resolution of the structures prevents a more precise evaluation of these changes [65,68].

191

192 ## Two distinct helicase loader complexes with a shared function

193 The BC and BP complexes reveal how the evolutionarily distinct structural
194 elements of DnaC and λ P converged on a common helicase-opening strategy [65,68].
195 In both loader complexes, the lasso/grappling hook segments of DnaC and λ P provide
196 key contacts to opening the DnaB helicase (**Figure 5**). Superposition of the two
197 complexes on a DnaB monomer reveals that the only segment in common between the
198 two loaders is a single α helix at the extreme amino-terminus of DnaC, or the carboxy-
199 terminus of λ P. In both complexes, this helix disrupts interactions between the LH linker
200 helix of one DnaB protomer and the DH docking helix on an adjacent subunit; each DnaB
201 protomer undergoes this interaction save for the one at the breach in the spiral. Insertion
202 of the loader α helix between the DnaB LH and DH elements reconfigures the CTD, and
203 concomitantly the NTD, tiers, from the closed planar to the open spiral form to allow
204 ssDNA to access the internal chamber of DnaB. Notably, in the BC structure, the N-
205 terminal lasso/grappling hook element represents the sole point of contact between DnaC
206 and DnaB; indeed, the isolated region harbors significant capacity to promote helicase
207 loading [66].

208 It was surprising to find that the AAA+ ATPase domains of DnaC make no contact
209 with DnaB, and thus, play no direct role in helicase opening (**Figure 3A**)[65]. By
210 comparison, the AAA+ ATPases of the evolutionarily related clamp loaders – which open
211 and chaperone the ring-shaped β and PCNA proteins onto DNA to aid polymerase
212 processivity – engage their client clamps directly [94–98]. For DnaC, the ATPase
213 elements appear to play a role in sensing the binding of ssDNA to the helicase and in
214 enhancing the efficiency of the DnaB-opening reaction [66]. AAA+ ATPases are often
215 pre-formed oligomers [99,100], unusually, in solution, DnaC is monomeric [54], however,
216 six copies oligomerize on DnaB in a manner stabilized by ATP [54,65]. Without ssDNA,
217 the nucleotide-binding sites of DnaC in the BC complex are filled with ATP and captured
218 in a configuration that is poised, but sub-optimal for catalysis. After sensing ssDNA, the
219 nucleotides sites on the DnaC oligomer are filled with ADP, as would be expected
220 following hydrolysis. ATP hydrolysis after ssDNA loading does not appear to allow DnaC
221 to dissociate from DnaB but may diminish stability of the DnaC oligomer. Biochemical
222 studies suggest that DnaG recruitment and primer synthesis are needed to promote loss
223 of DnaC from the complex [55,74,101].

224 In contrast to the modest interface between helicase and loader in the BC complex
225 [65,68], λ P forms an extensive interface with DnaB that encompasses both the globular
226 and lasso/grappling hook segments (**Figure 3B**). λ P is also a monomer in solution and
227 five copies assemble onto DnaB in the BP loader complex [68], however, few contacts
228 between loader subunits are seen. Inspection of the two loader complexes indicate that
229 the positioning of their C-terminal α helices between DnaB's LH and DH elements may
230 be sufficient for opening and that an extensive interface is dispensable for helicase

opening. This has been confirmed for DnaC [66], and we speculate that the extensive interface between the DnaB and the globular domain of λ P may form because of interacting with the opened helicase, rather than as its driver as previously proposed [68]. If so, then, what might be the functional role(s) for the extensive interface between λ P and DnaB? Biochemical studies provide a potential explanation for the structural dichotomy. It is known that λ P can displace DnaC from a preformed BC complex [58]; the extensive interface in the BP complex may aid displacement as part of a biological strategy to appropriate the host replication machinery away from the bacterial chromosome and toward the phage genome. Alternatively, the extensive interface in the BP complex may serve as a functional analogue to the extensive AAA+ interaction between DnaC globular domains in the BC complex. Regardless, in both the BC and BP complexes, overall stability is achieved by oligomerization, but by distinct means [65,68].

Although they feature some global architectural parallels, neither the globular domains of DnaC and λ P nor the extended lasso/grappling hook regions display any similarity in sequence [65,68]. Underscoring the dissimilarity is the opposing chain polarity of the grappling hook segments as they run across the surface of DnaB: DnaC runs N-to-C whereas λ P runs C-to-N (**Figures 3C-3D and Figure 5**). The finding that a single functionally significant α-helix in DnaC and λ P exhibits a divergent protein chain direction confirms their lack of evolutionary kinship and instead reflects a form of **molecular mimicry** that arose through **convergent evolution**. Molecular mimicry in bacterial DNA replication initiation joins other examples from protein synthesis [102–104], gene expression [105], apoptosis [106], host pathogen interactions [107–109], virally encoded proteins[110], and immunity and autoimmunity [111].

A recently described protein known as DciA serves as the primary helicase loader in bacteria that lack DnaC/DnaI

Outside of DnaC and λ P, Ferat and co-workers have reported that most bacteria lack homologs of DnaC (or the unrelated DnaI loader) and that helicase loading in these organisms instead appears to depend on a distinct protein called DciA [6] (**Table 1**). A structure of DciA from *Vibrio cholerae* (VcDciA) shows that the protein is composed of an ~110 aa N-terminal globular domain followed by a ~40 aa disordered C-terminal segment. Interestingly, the fold of the DciA globular domain is related to the N-terminal domain of the replication initiator, DnaA, as well as the C-terminal domain of the γ/τ/DnaX clamp loader subunit and the FliK flagellar hook-length control protein. VcDciA appears to stimulate the loading of the VcDnaB helicase onto DNA through a $DciA_3$:$DnaB_6$ intermediate; the LH-DH nexus that is targeted by DnaC and λ P has been suggested to serve as an important point of contact in this complex as well [9]. It has been proposed that VcDnaB may adopt an open spiral in solution and may harbor residues that specify loader preference. Given the widespread nature of the DciA system, additional chapters of the helicase loader story clearly remain to be written.

Concluding Remarks

In all cellular organisms, the regulated association of the replicative helicase with replication origins sets the stage for the initiation of DNA replication [1–8] (**Table 1** and **Figure 1**). However, significant differences are now evident in mechanisms by which

origin unwinding and helicase loading take place in bacteria as compared to archaea and eukaryotes. In bacterial replication systems, the current model holds that the initiator protein not only marks an origin for initiation, but also melts that origin, enabling the replicative helicase•loader complex to load onto the resultant ssDNA [8,12,13,31,112]. By contrast, in archaea and eukaryotes, the helicase is loaded by an initiator complex around duplex DNA, which is then subsequently melted by the helicase itself [113–118]. These distinct mechanisms are remarkable given that replication initiation machinery in all three domains of life is predicated on a related AAA+ fold [8,13,86]. Why the two approaches arose during evolution is unclear but may reflect an adaption to the two different families of hexameric helicases – one based on a RecA ATPase fold, and another based on a AAA+ ATPase domain [119–123] – that have been employed to support replication in bacteria as compared to archaea and eukaryotes. Structural analyses of two bacterial loaders bound to the *E. coli* DnaB helicase have for the first time illuminated the rich detail and diversity of helicase-ring opening as well as DNA association (**Figures 2, 3, 4 and Figure 5**). However, despite the insights gained from these models, several fundamental questions about replication initiation and helicase loading remain to be addressed (Outstanding Questions).

Acknowledgements

This work was supported by the National Science Foundation (DJ: MCB 1818255), the National Institutes of Health (DJ: R-01-GM084162 JMB: R37-071747), and the Department of Education (JC: PA200A150068).

Tables, Figures, and Figure legends

	E. coli	phage λ	*V. cholerae*
origin DNA	OriC	Oriλ	OriC-I/II
initiator	DnaA	O	DnaA/RctB
helicase	DnaB	DnaB	DnaB
loader	DnaC	P	DciA

Table 1. Molecules involved in various bacterial DNA replication initiation systems.

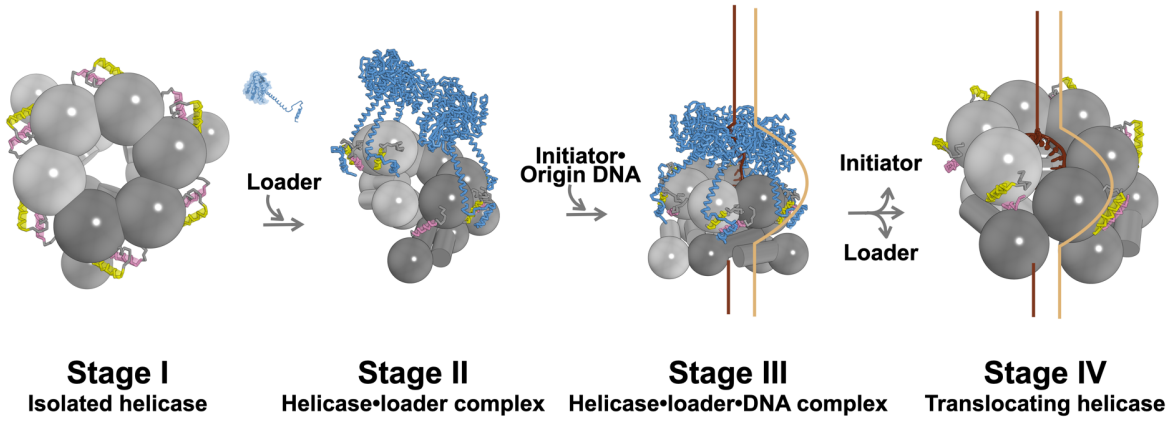

Figure 1. Loading of the Bacterial DnaB Replicative Helicase at a Replication Origin. The DnaB loading pathway passes through at least four stages (I, II, III, and IV). DnaB sub-domains are depicted according to their overall shape (amino-terminal domain (NTD): a mushroom-like shape; carboxy-terminal domain (CTD): sphere; both in varying shades of gray). The Linker-Helix (LH, pink) and Docking-Helix (DH, yellow) elements are depicted in a ribbon and transparent cylinder representation. The DnaC helicase loader is shown as a blue ribbon. The DNA strands, one of which is in included in the central DnaB chamber, and the second excluded, are colored in chocolate brown and light brown, respectively.

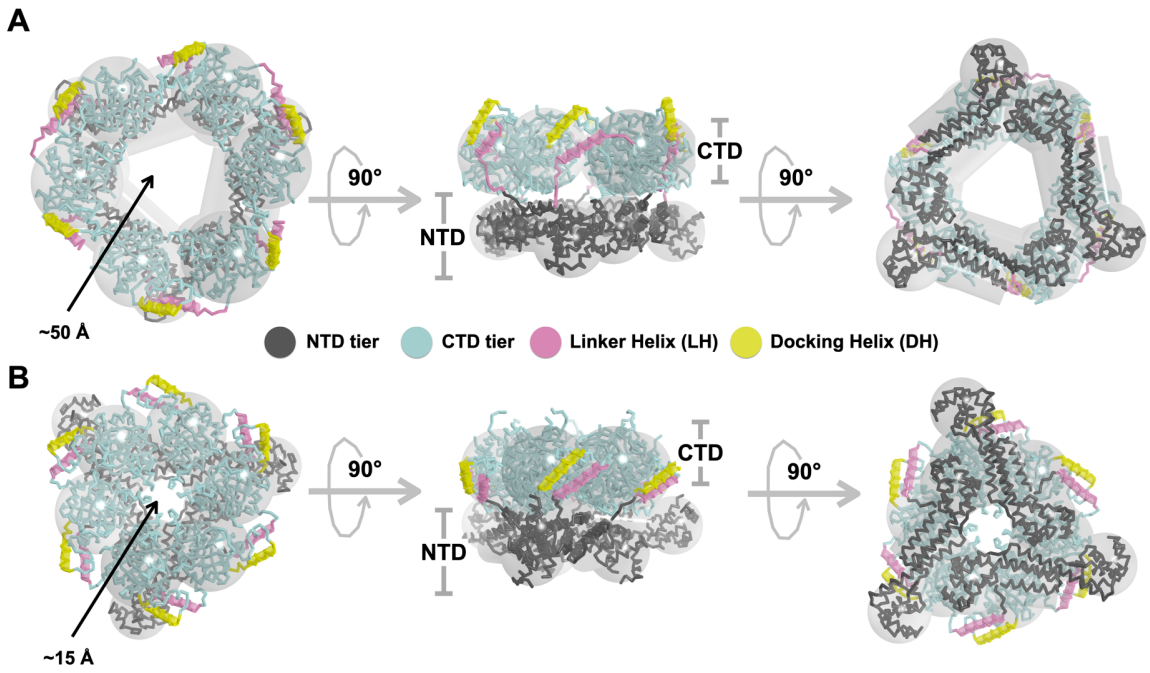

Figure 2. Overview of the DnaB replicative helicase. DnaB adopts at least two distinct configurations, termed dilated (A) and constricted (B) [62–64]**.** DnaB sub-domains are depicted according to their overall shape (amino-terminal domain (NTD): a mushroom-like shape; carboxy-terminal domain (CTD): sphere; both in varying shades of gray). Superimposed on these shapes of DnaB are ribbon representations, colored in gray and light cyan of the NTD and CTD tiers, respectively, in various poses of the dilated (A, PDB = 2R6A) and constricted (B, PDB = 4NMN) forms of DnaB. The linker and docking helices are depicted as cylinders, and colored pink and yellow, respectively.

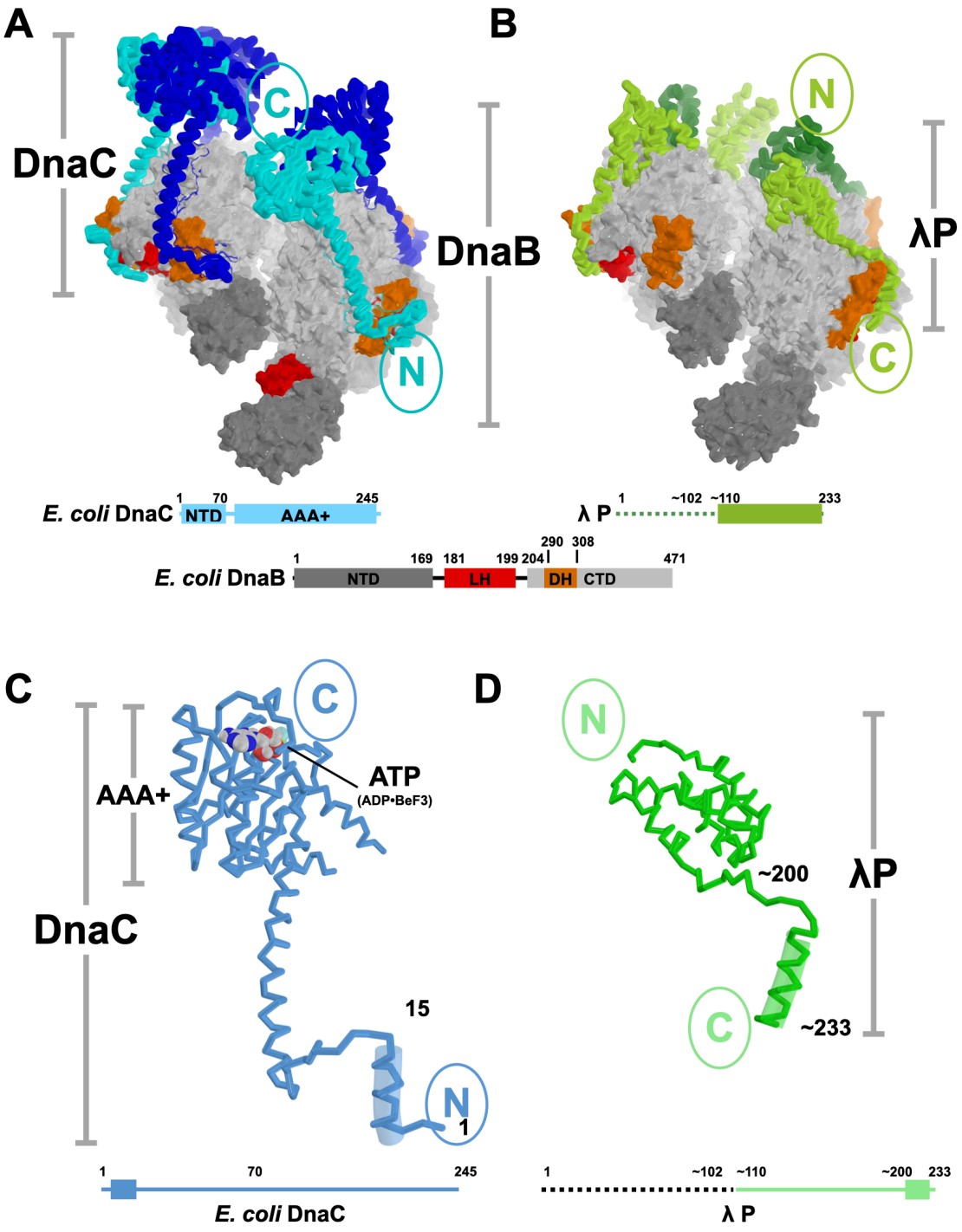

Figure 3. *E. coli* DnaB Complexed with the DnaC (A) and λ P (B) Helicase Loaders. Protomers of the DnaC and λ P helicase loaders are colored in alternating shades of blue (DnaC) and green (λ P). The *E. coli* DnaB helicase in each loader complex is represented in a surface rendering, with the amino-terminal domain (NTD) and the carboxy-terminal domain (CTD) layers colored in darker and lighter shades of gray, respectively. Linker-

helices (LH) and docking-helices (DH) are in colored in red and orange, respectively. The DnaB hexamers from the loader complexes are superimposed on the CTD of the protomer at the bottom of the spiral in this pose. The primary sequence of each loader and DnaB is represented as a linear schematic, with salient features annotated and colored to match the molecular representations. Only the carboxy terminal domain of λ P was visible in the EM maps of the BP complex (the missing segment is depicted as a dashed line). The terminal helix of each loader (DnaC: N-terminal; λ P: C-terminal) are depicted as ribbons and transparent cylinders. The amino (N) and carboxy (C) termini of each loader is indicated in each panel.

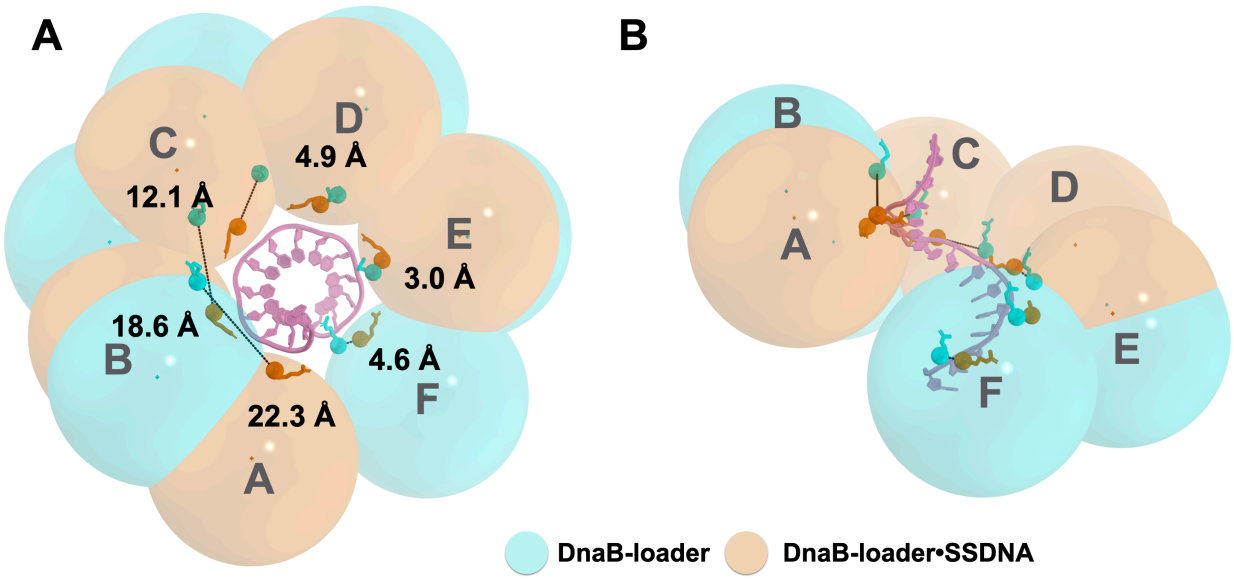

Figure 4. The ssDNA Binding Site is Altered in the Loader Bound Form of DnaB. The BC complex without ssDNA (PDB = 6QEL) was superimposed (RMSD = 0.85 Å) onto the C-terminal domain of chain F of the ssDNA bound complex (PDB = 6QEM). The large spheres represent the CTDs of the BC complex with (light brown) and without (cyan) ssDNA. The alpha carbons of arginine 403, which makes a key contact to ssDNA in the BC complex, are depicted as smaller spheres for the ssDNA complex (brown) and apo complex (dark cyan). Distances between the alpha carbons of arginine 403 from equivalent DnaB subunits are shown. The six chains from the ssDNA-bound complex are identified by letters (A, B, C, etc.). Poses in panels A and B are related by a 90° rotation about the horizontal axis.

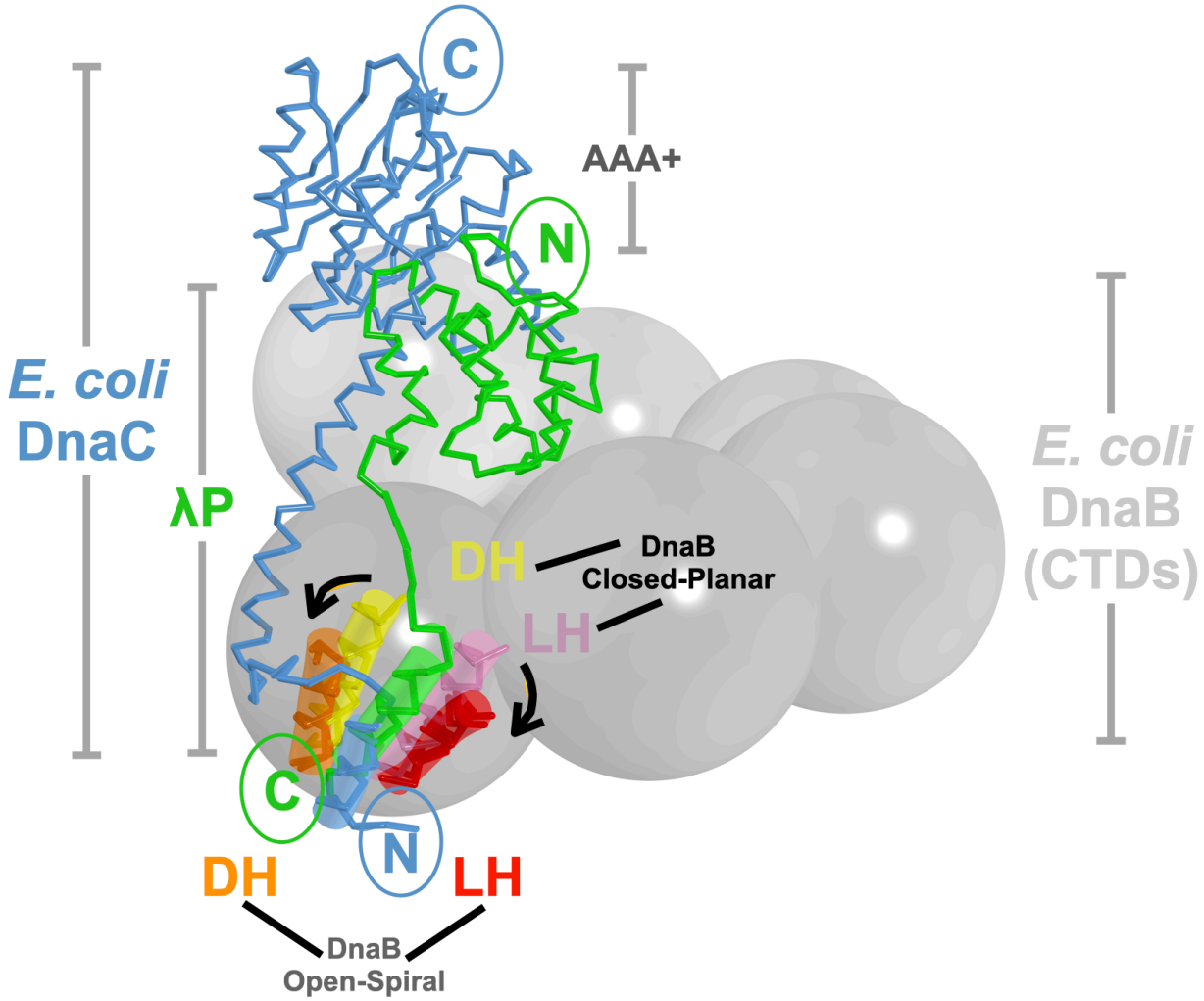

Figure 5. Convergent Evolution/Molecular Mimicry in the Mechanism of Opening of the *E. coli* DnaB Replicative Helicase by the *E. coli* DnaC and λ P Helicase Loaders. The DnaC and λ P helicase loaders are shown in a ribbon representation, colored light blue (DnaC) and green (λ P), with their respective amino and carboxy termini marked. For clarity, only one copy of each loader is shown. The DnaB hexamer from each loader complex is superimposed on the carboxy-terminal domain (CTD) of the protomer at the bottom of the spiral in this pose; the docking helix element was excluded from the alignment to produce an RMSD of 1.3 Å. For clarity, only the CTD tier of DnaB, represented as a set of spheres, colored in varying shades of gray, is shown. The linker (LH) and docking (DH) helices are depicted as a ribbon and transparent cylinder. The LH and DH from the closed-planar form of DnaB are colored yellow and pink, respectively; the corresponding elements from the DnaC and λ P loader-bound DnaB are in orange and red, respectively. The black arrow signifies direction of motion of the LH and DH elements on binding the DnaC and λ P loaders.

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Highlights

- The initiation of DNA replication is a tightly regulated process in all cellular domains of life and involves regulated recruitment and assembly of essential factors, including the replicative hexameric helicase complex, to replication origins.

- A crucial step during the replication initiation phase of DNA replication is loading of hexameric, ring-shaped replicative helicases onto DNA.

- In bacteria, the DnaB family of replicative helicases comprise six identical subunits which collectively create a central chamber to bind one of the ssDNA strands od dsDNA. The translocation of DnaB on ssDNA ahead of the DNA polymerase in the replisome separates the two strands to provide substrates for DNA synthesis.

- Recent structure determinations of two bacterial helicase loaders bound to the same DnaB helicase offers an opportunity to extract fundamental principles associated with DnaB opening and loading onto ssDNA.

- *E. coli* DnaC and bacteriophage λ P evolved independently to converge, through molecular mimicry, on a common helicase opening mechanism.

Outstanding Questions

- Is origin melting an emergent property that completely occurs completely through formation of the initiator•DNA complex?
- Does the DnaB bacterial replicative helicase also contribute to opening of the replication bubble as seen with the eukaryotic replicative helicase [117]?
- What mechanisms ensure the loading of two, and only two, helicases per initiation event?
- What mediates helicase loading in opposite orientations?
- What mechanisms promote the eviction of the helicase loaders?
- What, if any, of the mechanisms implemented by the DciA and DopE loader are in common with those used by DnaC or λ P?

Glossary

Replication origin – DNA sequence on a chromosome where DNA synthesis will begin. In bacteria, replication origins are up to hundreds of base-pairs in length and contain segments that are bound in a duplex state by the DnaA initiator protein, as well as segments that are melted (e.g., the DNA unwinding element (DUE) [124] DnaA trios [125] by the initiator [13,20,28,29].

DnaA – The bacterial replication initiator protein (*E. coli*: 467 amino acids) is comprised of four structural domains. Domain I harbors a K homology (KH) domain, domain II is a linker element, domain III encompasses the AAA+ ATPase functionality, and domain IV encodes a double-stranded helix-turn-helix DNA binding domain [10,12,30–32].

DnaB – The replicative helicase found in Gram-negative bacterial (*E. coli*: 471 amino acids) [4,12–14,50]. It is related to the DnaC helicase found in Gram-positive organisms.

DnaC – The replicative helicase loader found in certain Gram-negative bacteria (*E. coli*: 245 amino acids) [4,10,16,51–55]. This analog of this protein in Gram-positive species is DnaI.

Phage λ O – The replication initiator protein (299 amino acids) used by phage λ. λ O specifically recognizes a series of dsDNA binding sites in the phage λ replication origin [41–49].

Phage λ P – The helicase loader protein (233 amino acids) used by phage λ to assemble the DnaB helicase at the phage λ replication origin [43,45–48,56–59].

Replisome – A large (1-2 MDa) multi-protein complex that mediates synthesis of both strands (leading and lagging) of DNA. The replisome consists of 2-3 DNA polymerases, the replicative helicase, the sliding clamp, and the sliding clamp loader. Other proteins such as single-stranded DNA binding protein, gyrase, RNAse H, and DNA ligase interface with the replisome to support leading and lagging strand synthesis [126–129] .

Domain-swapped oligomer – An unusual architectural feature of some oligomeric protein ensembles wherein members of the assembly exchange a structural domain in a manner akin to a handshake between two persons. Swapping involves replacement of intra-monomer interactions between two sub-domains with nearly identical inter-monomer contacts. Such an oligomer becomes structurally intertwined because of the domain swapping [130–135].

Phosphate - Loop (P-loop) NTPases – Together with the Rossman fold family, P-loop NTPases encompass two major families of nucleotide handling proteins; proteins in this family couple the energy of nucleotide binding and hydrolysis to some chemical or mechanical transformation [123,136–138] . In concert with the crucial role played by ATP and other nucleotides in biology, P-loop NTPases family represent between 10 - 20% of all proteins in genomes in all cellular domains of life [137]. Members of this family share a conserved overall fold consisting of a four- or five-stranded beta-sheet sandwiched between two layers of alpha-helices;

this domain also exhibits two conserved amino acid sequence motifs termed the Walker A and Walker B (so named after John Walker, who first observed them in the F1 ATPases [139,140]). The Walker A sequence motif, which is the P-loop itself, is a glycine-rich loop terminated by a threonine or serine (GxxGxGK[T/S], where x = any residue); the backbone of this element makes a close approach to the β and γ phosphates of ATP, while the lysine and the threonine/serine contact the βphosphate and an associated Mg^{2+} ion, respectively. The Walker B sequence motif is a run of hydrophobic residues terminated by an aspartate residue (hhhhD; h = hydrophobic residue); the Walker B aspartate residue contributes to positioning the Mg ion and its associated water molecules. Sequence and structural analyses of P-loop NTPase family proteins highlight two major sub-divisions: the kinase – GTPase (KG) and the ASCE (additional strand catalytic glutamate) families. The ASCE grouping is further sub-classified into the RecA/F_1-F_o ATPases, AAA+ ATPases, ABC ATPases, nucleic acid helicases, PilT/FtsK ATPases, apoptotic NTPases, and the NACHT ATPases [123,137].

RecA-like ATPases – A sub-class of the ASCE sub-division of the P-loop NTPases. Members of this family adopt oligomeric configurations, and include the RecA recombinase, the DnaB replicative helicase, the F_1 sub-structure of ATP synthase, and the Rho helicase, [114,123,136,137]. All the elements found in the ASCE sub-class are seen in the RecA-like ATPases, as well as some additional structural elements. Amongst these is an arginine finger that enables stimulation of ATP hydrolysis *in trans* of nucleotide bound primarily by a neighboring subunit of the oligomer.

AAA+ ATPases – A sub-class of the ASCE sub-division of the P-loop NTPases. AAA+ (ATPases Associated with various Activities) are a large family of oligomeric, often ring-shaped, motors and switches with crucial functions in DNA replication, transcription, chaperones, proteases, and beyond [89,100,141]. This family of ATPases folds into a two-domain structure, one of which corresponds to the ASCE core domain (the second is a small helical domain). Residues and motifs conserved in this sub-class surround the general volume occupied by nucleotide. ATP binding sites are formed at subunit interfaces. Most of the contacts to bound nucleotide arise from one subunit at the interface, but the binding site is only completed by participation of residues (e.g., arginine finger) from a neighboring subunit.

Convergent evolution – a form of molecular evolution in which unrelated molecules independently evolve similar shapes or properties that reflect intrinsic structural or chemical constraints. Convergent evolution in the active sites of proteins has been documented in several enzymes [108]. It is axiomatic that a common ancestor is not present with examples of convergent evolution; by contrast, a common ancestor is an essential feature of divergent evolution [142,143].

Molecular mimicry – Close structural resemblance between two molecular entities. Mimicry can arise from divergent or convergent evolution [144].

Text Box:

Loading Bacterial Hexameric Replicative Helicases onto DNA

Ring-breakers – Loading factors that physically open hexameric helicase rings. *E. coli* DnaC and λ P are two examples of replicative helicase loaders that bind to a pre-formed closed DnaB ring and breach one its six subunit interfaces to enable ssDNA to enter an internal chamber.

Ring-makers – Loading factors that assemble helicase monomers into hexameric rings. *B. subtilis* DnaI is a bacterial helicase loader that is reported to operate in this manner.

Mechanisms of bacterial helicase loaders in the DciA/DopE families remain to be established. Distinct loading mechanisms with other hexameric helicases have also been described, including self-regulated ring closure for the transcription terminator Rho ATPase and chaperoned ring-closure for the MCM2-7 complex in eukaryotic DNA replication (reviewed in [114]).

Table

	E. coli	phage λ	*V. cholerae*
origin DNA	OriC	Oriλ	OriC-I/II
initiator	DnaA	O	DnaA/RctB
helicase	DnaB	DnaB	DnaB
loader	DnaC	P	DciA

Figure 1

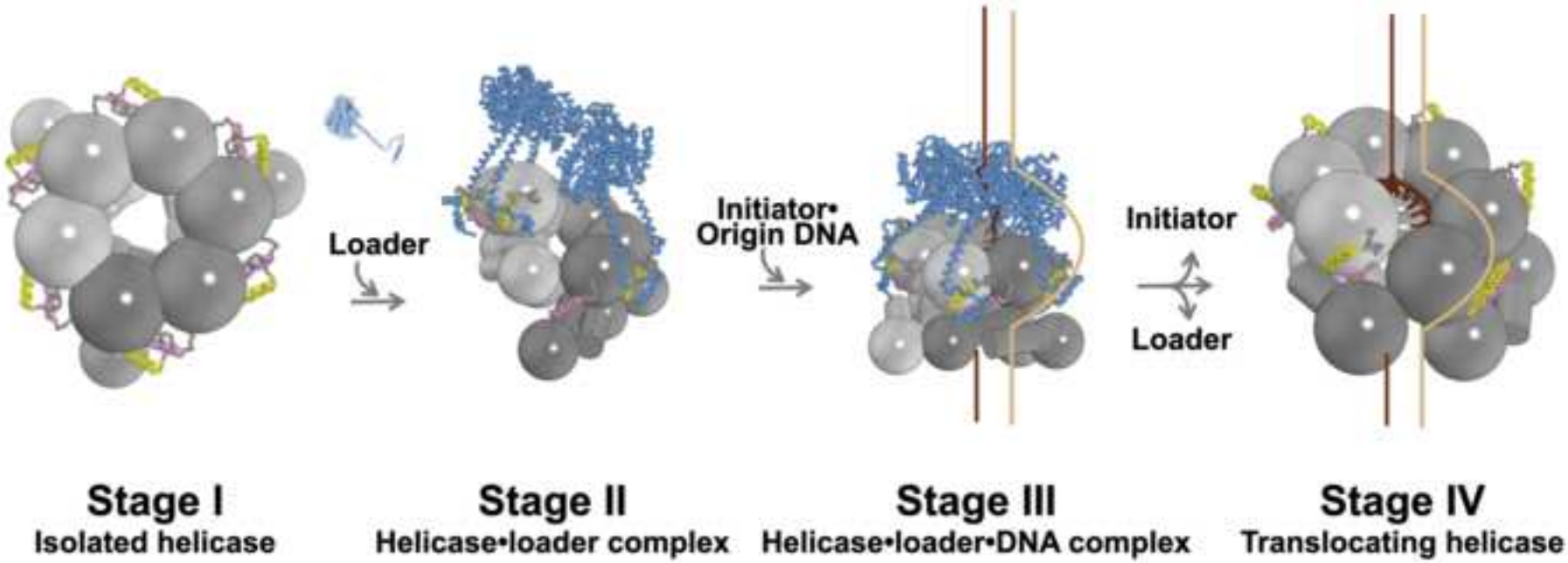


Figure 2

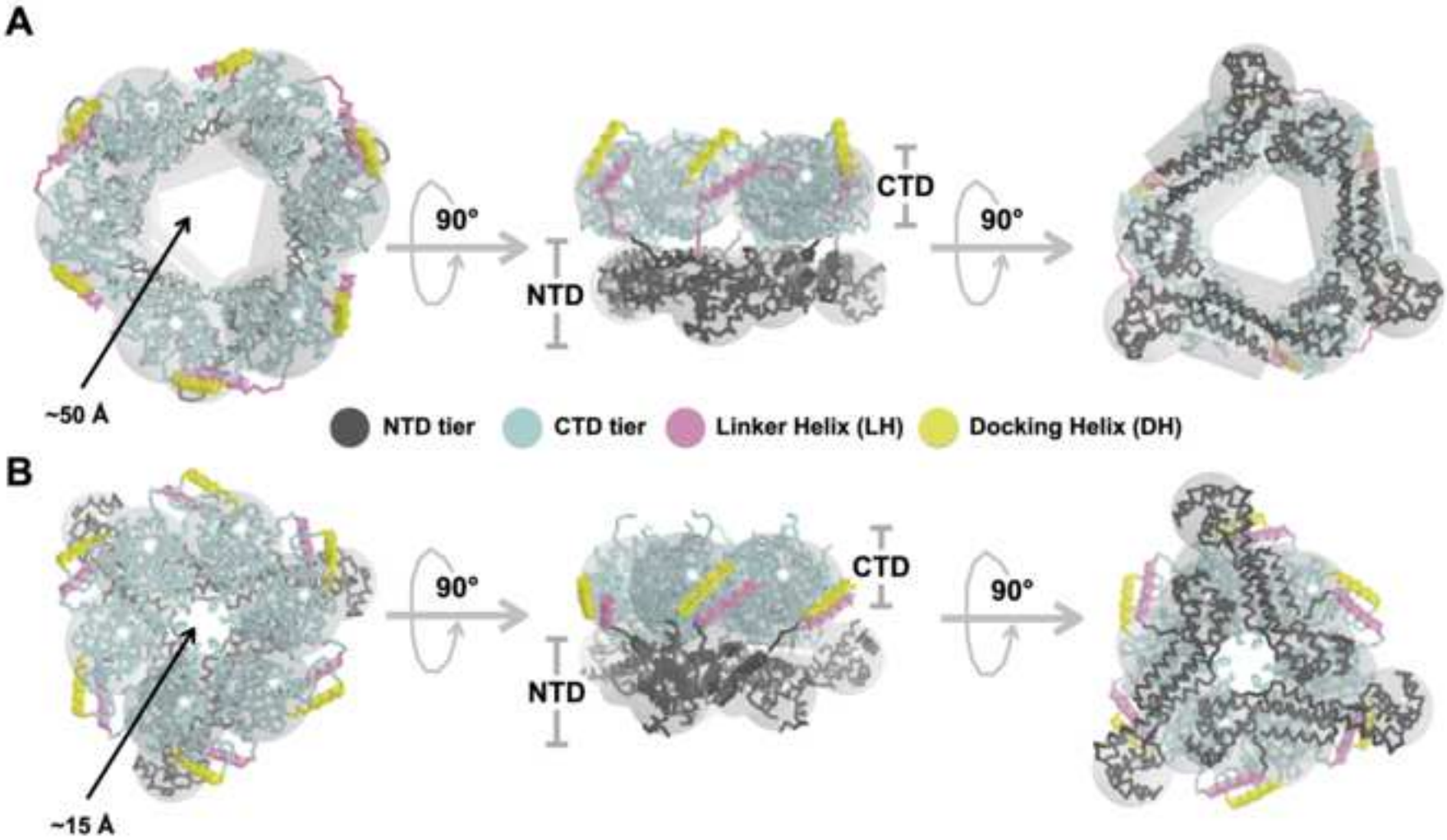


Figure 3

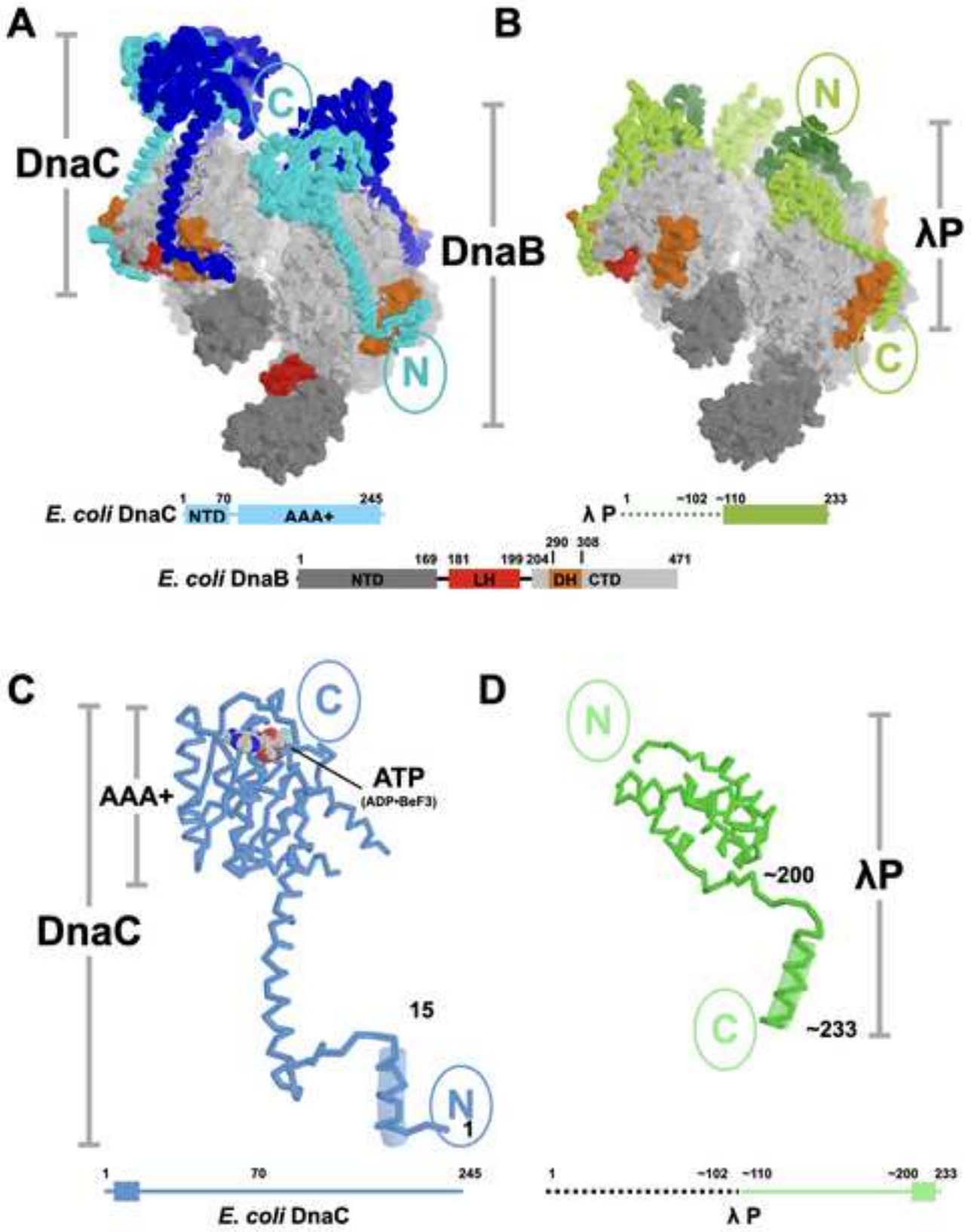


Figure 4

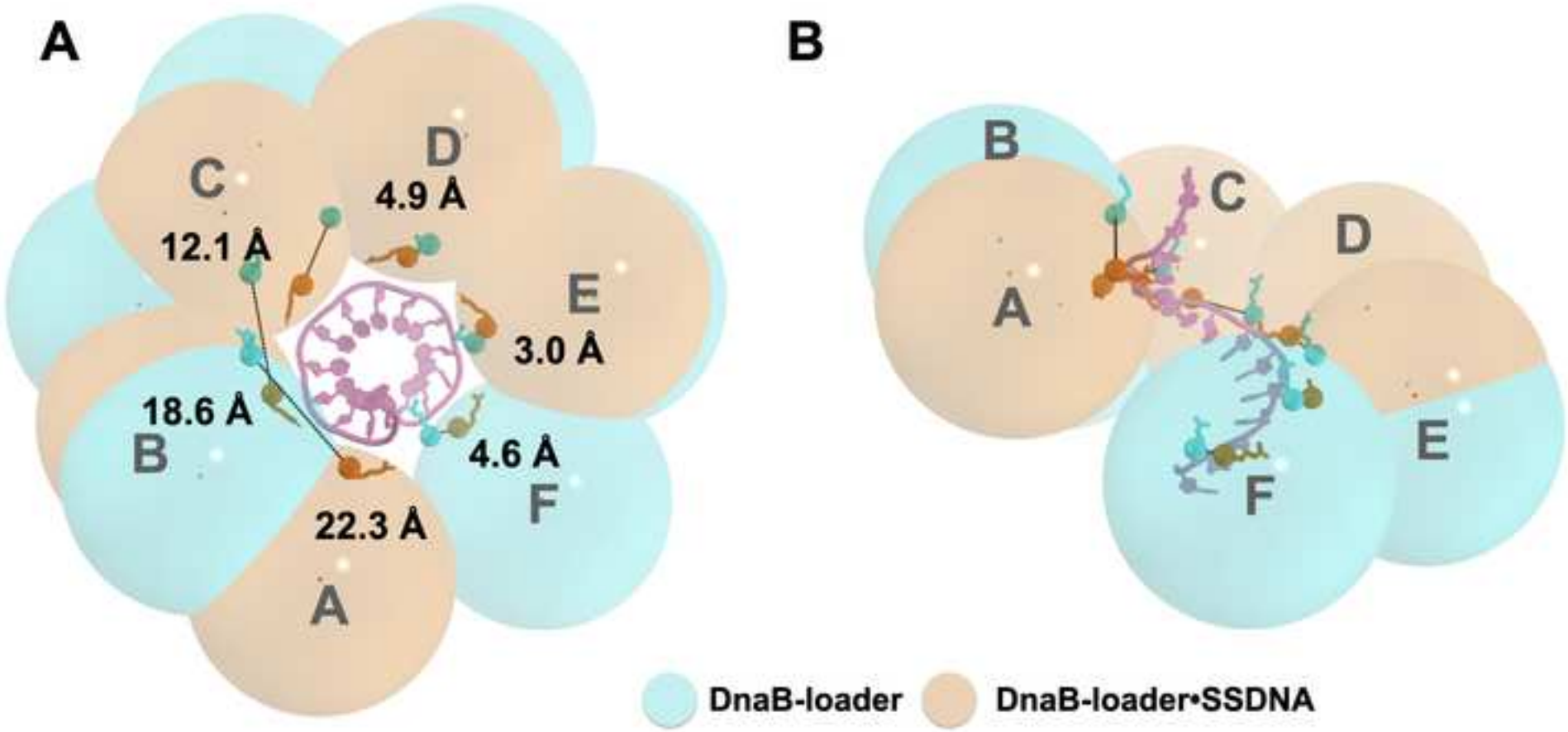
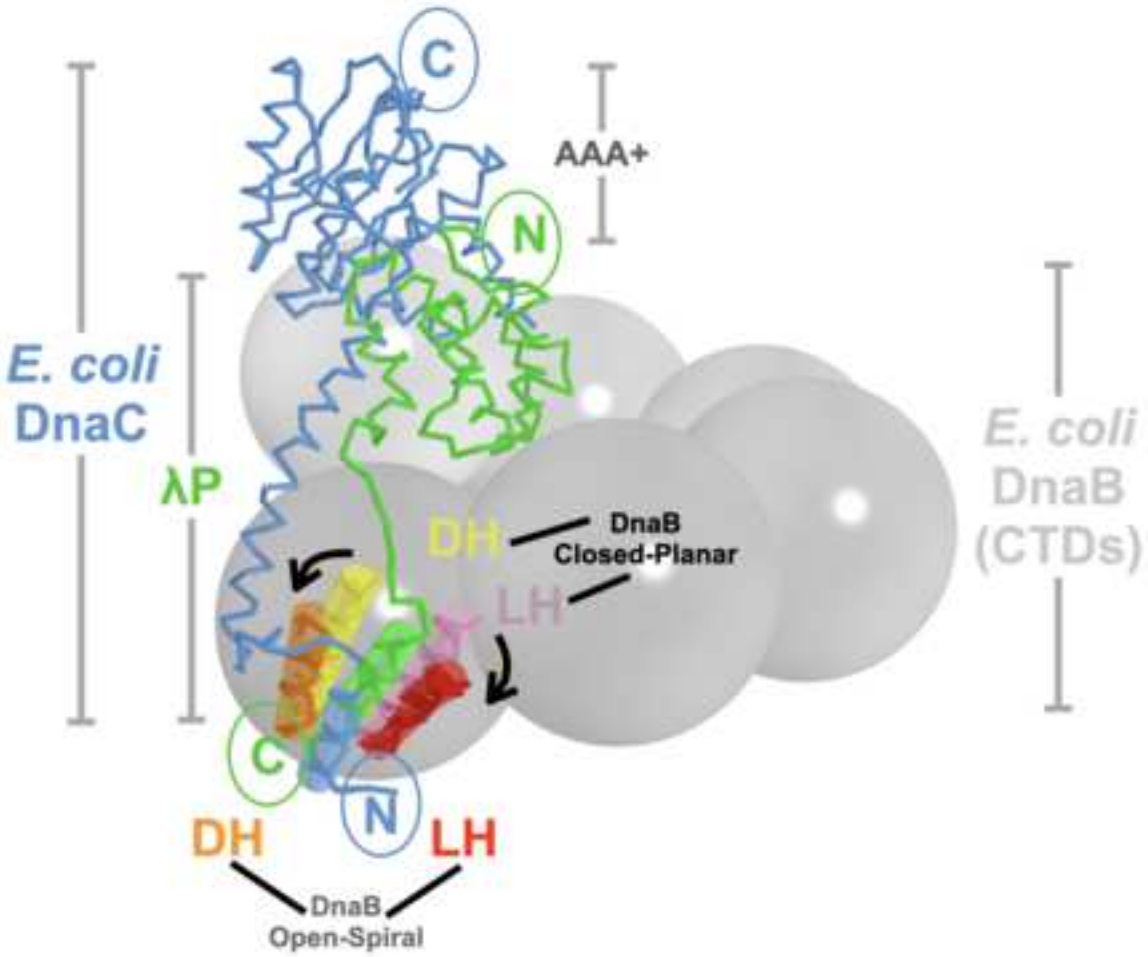


Figure 5



Convergent Evolution in Two Bacterial Replicative Helicase Loaders

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Abstract (120 words)

Dedicated loader proteins play essential roles in bacterial DNA replication by opening ring-shaped DnaB-family helicases and chaperoning ssDNA into a central motor chamber as a prelude to DNA unwinding. Although unrelated in sequence, the *E. coli* DnaC and bacteriophage λ P loaders feature a similar overall architecture: a globular domain linked to an extended lasso/grappling hook element, located at their amino and carboxy termini, respectively. Both loaders remodel a closed DnaB ring into nearly identical right-handed open conformations. The sole element shared by the loaders is a single alpha helix, which binds to the same site on the helicase. Physical features of the loaders establish that DnaC and λ P evolved independently to converge, through molecular mimicry, on a common helicase opening mechanism.

Keywords

DnaB, DnaC, λ P, DciA, DNA Replication, helicase loading, convergent evolution, molecular mimicry.

Introduction (~3100 words)

Specialized Factors Load the DnaB Bacterial Replicative Helicase onto the Replication Origin

The regulated loading of ring-shaped hexameric helicases onto chromosomal origins is an essential feature of DNA replication in all cellular domains of life [1–3]. Helicase deposition requires specialized factors known as helicase loaders, which operate during the initiation of DNA replication [4–8]. In bacteria, several helicase loaders have been studied, including *Escherichia coli* (*E. coli)* DnaC [4], bacteriophage λ P, *Bacillus subtilis* (*B. subtilis*) DnaI [5], and DciA/DopE, a recently described class of proteins which appear in bacteria that lack orthologs of DnaC or DnaI [6,7,9]. Helicase loading is also critical to assembly of eukaryal and archaeal **replisomes** [1,2,8].

The assembly of the bacterial replicative helicase (**Figure 1**), which is known as DnaB in most bacteria or DnaC in *B. subtilis* (henceforth called DnaB), onto origin DNA occurs during the initiation phase of DNA replication [8,10–14]. Our view of replication initiation in bacteria is informed by studies with primary and secondary chromosomes of bacteria, plasmids, and phages [15–27], and have implied the involvement of four classes of factors (**Table 1**): 1) a DNA sequence called a replication origin, where DNA synthesis will begin [13,20,28,29], 2) a replication initiator protein (*E. coli*: DnaA [10,12,30–32], *V. cholerae*: DnaA, RctB [18,21,23,25,33–37], plasmids: RepE, Pi, TrfA [38–40], phage lambda (λ): O [41–49]), 3) a DnaB-family replicative helicase [4,12–14,50], and, finally, 4) a helicase loader (*E. coli:* DnaC [4,10,16,51–55], phage λ: P [43,45–48,56–59], *V. cholerae*: DciA [6,9]). The multi-step process for initiating DNA replication begins with the recognition and binding of multiple copies of the initiator protein to dsDNA sites at the

replication origin; once bound, initiator proteins associate into a complex protein-DNA ensemble [4,10,12,19,31,60,61]. One of the outputs of the initiation phase of DNA replication is the melting of an A-T rich segment of the replication origin termed the DNA unwinding element (DUE) by the DnaA or the λ O initiator proteins [13,30,31], an event that provides single DNA strands (ssDNA) as substrates for DnaB loading. DnaB hexamers assemble into two-tiered rings formed by the amino (NTD) and carboxy-terminal domains (CTD) of the helicase; a so-termed 'linker helix' element (LH) connects these domains and packs against another alpha helix, termed the docking helix (DH), of a neighboring DnaB subunit to give rise to a **domain-swapped oligomer** [62–64] (**Figure 2**). The two DnaB tiers circumscribe an internal chamber into which one of the ssDNA strands from the replication origin will be loaded. One layer is formed out of six NTDs, which assemble into two different 'trimer-of-dimers' configurations that display pseudo-three-fold symmetry. These arrangements arise from alternative packing orientations for NTD dimers, which create several types of subunit interfaces of likely varying stability [62,65]. The CTD tier assembles out of six C-terminal domains (CTD) of DnaB, each of which harbors a **RecA-like ATPase domain**. In contrast to the NTD, the CTD layer exhibits a pseudo-six-fold arrangement, with a single type of interface.

Helicase loading onto ssDNA can be conceptually divided into four stages (**Figure 1**). During assembly, DnaB transitions between three conformations: closed planar, open right-handed spiral, and closed right-handed spiral [62–68]. In addition, the NTD and CTD layers of each of these conformers are found in one of two arrangements: dilated or constricted (**Figures 2A-2B**); these conformers differ on inter-protomer contacts. The isolated hexameric helicase, in both dilated and constricted closed-planar configurations,

74 populates Stage I. Formation of the helicase•helicase loader complex leads to Stage II,
75 while engagement of the helicase•loader complex with origin-derived ssDNA•initiator
76 (DnaA or λ O) populates Stage III. The ATPase and ssDNA translocation activities of the
77 helicase are suppressed during these latter two stages [56,58,69–71], with the
78 DnaA/replication origin complex also playing a role in loading or positioning the
79 helicase/loader complex using direct contacts between DnaA and DnaB [69–71] (the
80 involvement of contacts between other bacterial replication initiators and DnaB remains
81 to be clarified). Recently, cryogenic electron microscopy (cryo-EM) analyses of two
82 distinct bacterial helicase•loader complexes (*E. coli* DnaB•DnaC and *E. coli* DnaB•λP,
83 **Figure 1: Stages II-III, and Figure 3**) have shown that the helicase adopts an open right-
84 handed spiral configuration, promoted, and stabilized by interactions with the helicase
85 loaders [65,68]. The transition to Stage IV is accompanied by eviction of the loader and
86 initiator from the complex on DNA, which relieves inhibition of DnaB's activities. Notably,
87 in Stage III, the configuration of the DnaB-bound loader is nearly identical to the closed
88 spiral form seen in the loader-free helicase of Stage IV [65,67].

89 Helicase loading in bacteria occurs by one of at least two reported mechanisms
90 [72], termed: a) ring breaking, where DnaB hexamers are physically opened [73], and b)
91 ring making, in which hexamers are assembled [5]. It is now clear that both *E. coli* DnaC
92 and phage λ P are ring breakers: each loader binds to and delivers a pre-formed helicase
93 hexamer to its cognate origin [4,43,45–47,56,58,74]. DnaC and λ P are similar in that
94 both are essential for their respective organisms and both bind to ssDNA [56,58,75–77].
95 λ P also can displace DnaC from *E. coli* DnaB, implying that their respective binding sites
96 overlap [58,74]. These congruencies might imply a common ancestry; however, DnaC

and λP are unrelated in sequence and enzymatic function (e.g., DnaC is a known ATPase [10,53,75–77], whereas λ P is not [78]). Moreover, although each loading system requires ejection of the loader from the DNA complex for DnaB to transition to the translocation-competent form, eviction occurs by distinct mechanisms. For DnaC, nucleotide dynamics in its **AAA+ ATPase domain**, along with RNA synthesis by the DnaG primase, are significant features in eviction [10,52,53,76,77,79]. By contrast, removal of λ P requires the host DnaK/DnaJ/GrpE chaperone machinery [31–34]. Helicase loading is also a feature during restart of DNA replication after it has prematurely been halted in response to DNA damage and involves a distinct set of proteins (PriA, PriB, PriC, and DnaT); the reader is referred to the literature for a more complete treatment of helicase loading during replication restart [80–82].

Here, we compare recent structures of two bacterial helicase loaders: *E. coli* DnaC [65] and phage λ P [68] bound to the same *E. coli* DnaB helicase (**Figure 3**). This comparison provides an opportunity to understand the mechanisms of the two loaders and to extract central principles associated with DnaB opening and loading onto ssDNA. A recent crystal structure of the interaction domains of DnaB and DnaC [83] comports with the EM analyses of the complete complexes. A new study of DciA•DnaB interactions also points to some conserved elements of helicase opening in this system as well [9]. Further study will be required to establish the mechanistic relationships, if any, between the DciA and *B. subtilis* DnaI/DnaB/DnaD loaders [5,84,85] and the better understood *E. coli* DnaC [65] and λ P [68] systems.

Though unrelated by sequence and fold, *E. coli* DnaC and λ P exhibit analogous global architectures

Inspection of the *E. coli* DnaB•DnaC (BC) and the *E. coli* DnaB•phage λ P (BP) complexes shows that the two loaders engage the carboxy-terminal (CTD) ATPase surface of DnaB to form a three-layered ensemble [65,68,83]. One layer corresponds to an oligomeric form of the helicase loader (hexameric for DnaC and pentameric for λ P), while the second and third correspond to the NTD and CTD tiers of DnaB (**Figures 3A-B**). Although six copies of DnaC are present in the *E. coli* BC complex and five λ P protomers are found in the BP assembly, the stoichiometries seen in the cryo-EM structures do not necessarily preclude the possibility that complexes with fewer copies of the loader might be active in supporting helicase loading. Significantly, each loader adopts a distinct and unrelated structure, yet the monomers of both exhibit a similar overall architecture: a globular domain fused to an extended segment that forms a lasso/grappling hook element (**Figures 3C-D**).

The globular domain of DnaC consists of an AAA+ ATPase module that is fused to a ~75 residue N-terminal segment (**Figure 3C**)[65,83]. The amino-terminal segment of DnaC consists of a long α-helix that extends along the CTD of a DnaB protomer and initiates from a helix-loop-helix element that packs against the LH linker helix from one DnaB subunit and the DH docking helix from another. Notably, the N-terminal segment provides the only contacts between DnaC and the DnaB helicase. The six copies of the globular DnaC AAA+ domain assemble into an open spiral like that seen in related ATPases such as DnaA and archaeal/eukaryal MCM helicases [86–92]. In the absence of ssDNA, five of the six nucleotide-binding sites in DnaC are populated with an ATP

analog (ADP•BeF_3), whereas the sixth (which sits at the gap in the DnaC spiral) engages ADP, likely because its catalytic center lacks important functional contacts from a neighboring protomer. Rationalizing this arrangement of nucleotides is the prior finding that the ATP form of DnaC suppresses DnaB's helicase activity and as it stabilizes the ssDNA complex [52].

For the λ P loader, only the C-terminal ~125 residues were resolved in EM density maps [68]. This region consists of an α-helical globular domain fused to an extended segment of ~45 residues (**Figure 3D**). The λ P extension forms a sub-structure analogous to, but distinct from, that seen in DnaC [65,83], terminating in a single α-helix that packs against the LH and DH elements of two adjacent subunits of DnaB. Both the globular domain and the grappling hook/lasso segment of λ P contact two consecutive subunits of the DnaB hexamer. These interactions are repeated in the five copies of λ P in the BP complex to create an open helical arrangement of loaders; the breached interface of the DnaB hexamer precludes binding of a sixth copy of λ P. The low resolution (4.1 Å) of the BP EM maps in the region of the loader limits analysis of interfaces between λ P protomers; nevertheless, an extensive interface between the five λ P protomers does not appear to form.

E. coli DnaC and phage λP reconfigure DnaB into an open right-handed spiral

Despite their evolutionarily distinct structures and contacts with DnaB, both DnaC and λ P reconfigure the helicase into highly similar, open-spiral configurations (root mean square deviation (RMSD) of ~2.2 Å, calculated from 2611 Cα positions that span the

DnaB hexamer) [65,68]. The DnaB NTDs also both adopt a constricted configuration, albeit with a spiral (as opposed to planar) shape that bears a split between one of the subunit interfaces. The similarity between DnaB in the two loader complexes is also evident from the average helical pitch and twist values of the open spirals in both the NTD (~2.8 Å and 60.0° for BC *vs*. ~2.6 Å and 59.9° for BP) and CTD layers (~19.3 Å and ~55.3° for BC *vs*. ~16 Å and ~56° for BP). Changes induced by each loader rupture one of the DnaB subunit interfaces at both the NTD and CTD layers to create openings (15-20 Å) of sufficient size to allow ssDNA access to the internal chamber.

Changes to the helical pitch and twist of the DnaB hexamer within each loader complex combine to alter the configuration of the ssDNA-binding site in the helicase. Superposition of DnaB from each loader complex reveals significant changes in the position of DNA binding residues in comparison to that when DnaB is bound to ssDNA (**Figure 4)** [65]. When bound to ssDNA and the loader, the CTD of each DnaB protomer projects three residues (*E. coli*: R403, E404, G406) on a DNA binding loop into the helicase pore to contact ssDNA. In the loader-only complexes, reconfiguration of the CTD layer shifts the positions of the alpha-carbons of these residues by ~10-30 Å (in BC) or ~5-20 Å (in BP).

The disposition and nucleotide occupancy of the six RecA-type ATPase sites in DnaB are also altered in the complexes with DnaC and λ P [65,68]. ATPase activity by DnaB relies on 'composite' nucleotide binding sites, wherein residues from two adjacent subunits contribute to a single catalytic center [63,64,67,93]. In both helicase•loader complexes, five of the six ATPase sites in DnaB are occupied by ADP, while the sixth, which sits at the breach in the CTD ring, is vacant; when bound to just ssDNA, this

187 constellation of sites are filled with a nucleoside triphosphate analog (ADP•BeF_3) instead.
188 The alterations in CTD orientation appear to have remodeled the five ADP-filled sites of
189 the loader-bound helicase into non-optimal catalytic configurations as well, although the
190 resolution of the structures prevents a more precise evaluation of these changes [65,68].

191

192 ## Two distinct helicase loader complexes with a shared function

193 The BC and BP complexes reveal how the evolutionarily distinct structural
194 elements of DnaC and λ P converged on a common helicase-opening strategy [65,68].
195 In both loader complexes, the lasso/grappling hook segments of DnaC and λ P provide
196 key contacts to opening the DnaB helicase (**Figure 5**). Superposition of the two
197 complexes on a DnaB monomer reveals that the only segment in common between the
198 two loaders is a single α helix at the extreme amino-terminus of DnaC, or the carboxy-
199 terminus of λ P. In both complexes, this helix disrupts interactions between the LH linker
200 helix of one DnaB protomer and the DH docking helix on an adjacent subunit; each DnaB
201 protomer undergoes this interaction save for the one at the breach in the spiral. Insertion
202 of the loader α helix between the DnaB LH and DH elements reconfigures the CTD, and
203 concomitantly the NTD, tiers, from the closed planar to the open spiral form to allow
204 ssDNA to access the internal chamber of DnaB. Notably, in the BC structure, the N-
205 terminal lasso/grappling hook element represents the sole point of contact between DnaC
206 and DnaB; indeed, the isolated region harbors significant capacity to promote helicase
207 loading [66].

208 It was surprising to find that the AAA+ ATPase domains of DnaC make no contact
209 with DnaB, and thus, play no direct role in helicase opening (**Figure 3A**)[65]. By
210 comparison, the AAA+ ATPases of the evolutionarily related clamp loaders – which open
211 and chaperone the ring-shaped β and PCNA proteins onto DNA to aid polymerase
212 processivity – engage their client clamps directly [94–98]. For DnaC, the ATPase
213 elements appear to play a role in sensing the binding of ssDNA to the helicase and in
214 enhancing the efficiency of the DnaB-opening reaction [66]. AAA+ ATPases are often
215 pre-formed oligomers [99,100], unusually, in solution, DnaC is monomeric [54], however,
216 six copies oligomerize on DnaB in a manner stabilized by ATP [54,65]. Without ssDNA,
217 the nucleotide-binding sites of DnaC in the BC complex are filled with ATP and captured
218 in a configuration that is poised, but sub-optimal for catalysis. After sensing ssDNA, the
219 nucleotides sites on the DnaC oligomer are filled with ADP, as would be expected
220 following hydrolysis. ATP hydrolysis after ssDNA loading does not appear to allow DnaC
221 to dissociate from DnaB but may diminish stability of the DnaC oligomer. Biochemical
222 studies suggest that DnaG recruitment and primer synthesis are needed to promote loss
223 of DnaC from the complex [55,74,101].

224 In contrast to the modest interface between helicase and loader in the BC complex
225 [65,68], λ P forms an extensive interface with DnaB that encompasses both the globular
226 and lasso/grappling hook segments (**Figure 3B**). λ P is also a monomer in solution and
227 five copies assemble onto DnaB in the BP loader complex [68], however, few contacts
228 between loader subunits are seen. Inspection of the two loader complexes indicate that
229 the positioning of their C-terminal α helices between DnaB's LH and DH elements may
230 be sufficient for opening and that an extensive interface is dispensable for helicase

opening. This has been confirmed for DnaC [66], and we speculate that the extensive interface between the DnaB and the globular domain of λ P may form because of interacting with the opened helicase, rather than as its driver as previously proposed [68]. If so, then, what might be the functional role(s) for the extensive interface between λ P and DnaB? Biochemical studies provide a potential explanation for the structural dichotomy. It is known that λ P can displace DnaC from a preformed BC complex [58]; the extensive interface in the BP complex may aid displacement as part of a biological strategy to appropriate the host replication machinery away from the bacterial chromosome and toward the phage genome. Alternatively, the extensive interface in the BP complex may serve as a functional analogue to the extensive AAA+ interaction between DnaC globular domains in the BC complex. Regardless, in both the BC and BP complexes, overall stability is achieved by oligomerization, but by distinct means [65,68].

Although they feature some global architectural parallels, neither the globular domains of DnaC and λ P nor the extended lasso/grappling hook regions display any similarity in sequence [65,68]. Underscoring the dissimilarity is the opposing chain polarity of the grappling hook segments as they run across the surface of DnaB: DnaC runs N-to-C whereas λ P runs C-to-N (**Figures 3C-3D and Figure 5**). The finding that a single functionally significant α-helix in DnaC and λ P exhibits a divergent protein chain direction confirms their lack of evolutionary kinship and instead reflects a form of **molecular mimicry** that arose through **convergent evolution**. Molecular mimicry in bacterial DNA replication initiation joins other examples from protein synthesis [102–104], gene expression [105], apoptosis [106], host pathogen interactions [107–109], virally encoded proteins[110], and immunity and autoimmunity [111].

A recently described protein known as DciA serves as the primary helicase loader in bacteria that lack DnaC/DnaI

Outside of DnaC and λ P, Ferat and co-workers have reported that most bacteria lack homologs of DnaC (or the unrelated DnaI loader) and that helicase loading in these organisms instead appears to depend on a distinct protein called DciA [6] (**Table 1**). A structure of DciA from *Vibrio cholerae* (VcDciA) shows that the protein is composed of an ~110 aa N-terminal globular domain followed by a ~40 aa disordered C-terminal segment. Interestingly, the fold of the DciA globular domain is related to the N-terminal domain of the replication initiator, DnaA, as well as the C-terminal domain of the γ/τ/DnaX clamp loader subunit and the FliK flagellar hook-length control protein. VcDciA appears to stimulate the loading of the VcDnaB helicase onto DNA through a $DciA_3$:$DnaB_6$ intermediate; the LH-DH nexus that is targeted by DnaC and λ P has been suggested to serve as an important point of contact in this complex as well [9]. It has been proposed that VcDnaB may adopt an open spiral in solution and may harbor residues that specify loader preference. Given the widespread nature of the DciA system, additional chapters of the helicase loader story clearly remain to be written.

Concluding Remarks

In all cellular organisms, the regulated association of the replicative helicase with replication origins sets the stage for the initiation of DNA replication [1–8] (**Table 1** and **Figure 1**). However, significant differences are now evident in mechanisms by which

origin unwinding and helicase loading take place in bacteria as compared to archaea and eukaryotes. In bacterial replication systems, the current model holds that the initiator protein not only marks an origin for initiation, but also melts that origin, enabling the replicative helicase•loader complex to load onto the resultant ssDNA [8,12,13,31,112]. By contrast, in archaea and eukaryotes, the helicase is loaded by an initiator complex around duplex DNA, which is then subsequently melted by the helicase itself [113–118]. These distinct mechanisms are remarkable given that replication initiation machinery in all three domains of life is predicated on a related AAA+ fold [8,13,86]. Why the two approaches arose during evolution is unclear but may reflect an adaption to the two different families of hexameric helicases – one based on a RecA ATPase fold, and another based on a AAA+ ATPase domain [119–123] – that have been employed to support replication in bacteria as compared to archaea and eukaryotes. Structural analyses of two bacterial loaders bound to the *E. coli* DnaB helicase have for the first time illuminated the rich detail and diversity of helicase-ring opening as well as DNA association (**Figures 2, 3, 4 and Figure 5**). However, despite the insights gained from these models, several fundamental questions about replication initiation and helicase loading remain to be addressed (Outstanding Questions).

Glossary

Replication origin – DNA sequence on a chromosome where DNA synthesis will begin. In bacteria, replication origins are up to hundreds of base-pairs in length and contain segments that are bound in a duplex state by the DnaA initiator protein, as well as segments that are melted (e.g., the DNA unwinding element (DUE) [124] DnaA trios [125] by the initiator [13,20,28,29].

DnaA – The bacterial replication initiator protein (*E. coli*: 467 amino acids) is comprised of four structural domains. Domain I harbors a K homology (KH) domain, domain II is a linker element, domain III encompasses the AAA+ ATPase functionality, and domain IV encodes a double-stranded helix-turn-helix DNA binding domain [10,12,30–32].

DnaB – The replicative helicase found in Gram-negative bacterial (*E. coli*: 471 amino acids) [4,12–14,50]. It is related to the DnaC helicase found in Gram-positive organisms.

DnaC – The replicative helicase loader found in certain Gram-negative bacteria (*E. coli*: 245 amino acids) [4,10,16,51–55]. This analog of this protein in Gram-positive species is DnaI.

Phage λ O – The replication initiator protein (299 amino acids) used by phage λ. λ O specifically recognizes a series of dsDNA binding sites in the phage λ replication origin [41–49].

Phage λ P – The helicase loader protein (233 amino acids) used by phage λ to assemble the DnaB helicase at the phage λ replication origin [43,45–48,56–59].

Replisome – A large (1-2 MDa) multi-protein complex that mediates synthesis of both strands (leading and lagging) of DNA. The replisome consists of 2-3 DNA polymerases, the replicative helicase, the sliding clamp, and the sliding clamp loader. Other proteins such as single-stranded DNA binding protein, gyrase, RNAse H, and DNA ligase interface with the replisome to support leading and lagging strand synthesis [126–129] .

Domain-swapped oligomer – An unusual architectural feature of some oligomeric protein ensembles wherein members of the assembly exchange a structural domain in a manner akin to a handshake between two persons. Swapping involves replacement of intra-monomer interactions between two sub-domains with nearly identical inter-monomer contacts. Such an oligomer becomes structurally intertwined because of the domain swapping [130–135].

Phosphate - Loop (P-loop) NTPases – Together with the Rossman fold family, P-loop NTPases encompass two major families of nucleotide handling proteins; proteins in this family couple the energy of nucleotide binding and hydrolysis to some chemical or mechanical transformation [123,136–138] . In concert with the crucial role played by ATP and other nucleotides in biology, P-loop NTPases family represent between 10 - 20% of all proteins in genomes in all cellular domains of life [137]. Members of this family share a conserved overall fold consisting of a four- or five-stranded beta-sheet sandwiched between two layers of alpha-helices;

this domain also exhibits two conserved amino acid sequence motifs termed the Walker A and Walker B (so named after John Walker, who first observed them in the F1 ATPases [139,140]). The Walker A sequence motif, which is the P-loop itself, is a glycine-rich loop terminated by a threonine or serine (GxxGxGK[T/S], where x = any residue); the backbone of this element makes a close approach to the β and γ phosphates of ATP, while the lysine and the threonine/serine contact the βphosphate and an associated Mg^{2+} ion, respectively. The Walker B sequence motif is a run of hydrophobic residues terminated by an aspartate residue (hhhhD; h = hydrophobic residue); the Walker B aspartate residue contributes to positioning the Mg ion and its associated water molecules. Sequence and structural analyses of P-loop NTPase family proteins highlight two major sub-divisions: the kinase – GTPase (KG) and the ASCE (additional strand catalytic glutamate) families. The ASCE grouping is further sub-classified into the RecA/F_1-F_o ATPases, AAA+ ATPases, ABC ATPases, nucleic acid helicases, PilT/FtsK ATPases, apoptotic NTPases, and the NACHT ATPases [123,137].

RecA-like ATPases – A sub-class of the ASCE sub-division of the P-loop NTPases. Members of this family adopt oligomeric configurations, and include the RecA recombinase, the DnaB replicative helicase, the F_1 sub-structure of ATP synthase, and the Rho helicase, [114,123,136,137]. All the elements found in the ASCE sub-class are seen in the RecA-like ATPases, as well as some additional structural elements. Amongst these is an arginine finger that enables stimulation of ATP hydrolysis *in trans* of nucleotide bound primarily by a neighboring subunit of the oligomer.

AAA+ ATPases – A sub-class of the ASCE sub-division of the P-loop NTPases. AAA+ (ATPases Associated with various Activities) are a large family of oligomeric, often ring-shaped, motors and switches with crucial functions in DNA replication, transcription, chaperones, proteases, and beyond [89,100,141]. This family of ATPases folds into a two-domain structure, one of which corresponds to the ASCE core domain (the second is a small helical domain). Residues and motifs conserved in this sub-class surround the general volume occupied by nucleotide. ATP binding sites are formed at subunit interfaces. Most of the contacts to bound nucleotide arise from one subunit at the interface, but the binding site is only completed by participation of residues (e.g., arginine finger) from a neighboring subunit.

Convergent evolution – a form of molecular evolution in which unrelated molecules independently evolve similar shapes or properties that reflect intrinsic structural or chemical constraints. Convergent evolution in the active sites of proteins has been documented in several enzymes [108]. It is axiomatic that a common ancestor is not present with examples of convergent evolution; by contrast, a common ancestor is an essential feature of divergent evolution [142,143].

Molecular mimicry – Close structural resemblance between two molecular entities. Mimicry can arise from divergent or convergent evolution [144].

Text Box:

Loading Bacterial Hexameric Replicative Helicases onto DNA

Ring-breakers – Loading factors that physically open hexameric helicase rings. *E. coli* DnaC and λ P are two examples of replicative helicase loaders that bind to a pre-formed closed DnaB ring and breach one its six subunit interfaces to enable ssDNA to enter an internal chamber.

Ring-makers – Loading factors that assemble helicase monomers into hexameric rings. *B. subtilis* DnaI is a bacterial helicase loader that is reported to operate in this manner.

Mechanisms of bacterial helicase loaders in the DciA/DopE families remain to be established. Distinct loading mechanisms with other hexameric helicases have also been described, including self-regulated ring closure for the transcription terminator Rho ATPase and chaperoned ring-closure for the MCM2-7 complex in eukaryotic DNA replication (reviewed in [114]).

Highlights

- The initiation of DNA replication is a tightly regulated process in all cellular domains of life and involves regulated recruitment and assembly of essential factors, including the replicative hexameric helicase complex, to replication origins.

- A crucial step during the replication initiation phase of DNA replication is loading of hexameric, ring-shaped replicative helicases onto DNA.

- In bacteria, the DnaB family of replicative helicases comprise six identical subunits which collectively create a central chamber to bind one of the ssDNA strands od dsDNA. The translocation of DnaB on ssDNA ahead of the DNA polymerase in the replisome separates the two strands to provide substrates for DNA synthesis.

- Recent structure determinations of two bacterial helicase loaders bound to the same DnaB helicase offers an opportunity to extract fundamental principles associated with DnaB opening and loading onto ssDNA.

- *E. coli* DnaC and bacteriophage λ P evolved independently to converge, through molecular mimicry, on a common helicase opening mechanism.

Outstanding Questions

- Is origin melting an emergent property that completely occurs completely through formation of the initiator•DNA complex?
- Does the DnaB bacterial replicative helicase also contribute to opening of the replication bubble as seen with the eukaryotic replicative helicase [117]?
- What mechanisms ensure the loading of two, and only two, helicases per initiation event?
- What mediates helicase loading in opposite orientations?
- What mechanisms promote the eviction of the helicase loaders?
- What, if any, of the mechanisms implemented by the DciA and DopE loader are in common with those used by DnaC or λ P?

Acknowledgements

This work was supported by the National Science Foundation (DJ: MCB 1818255), the National Institutes of Health (DJ: R-01-GM084162 JMB: R37-071747), and the Department of Education (JC: PA200A150068).

Figure legends

	E. coli	phage λ	*V. cholerae*
origin DNA	OriC	Oriλ	OriC-I/II
initiator	DnaA	O	DnaA/RctB
helicase	DnaB	DnaB	DnaB
loader	DnaC	P	DciA

Table 1. Molecules involved in various bacterial DNA replication initiation systems.

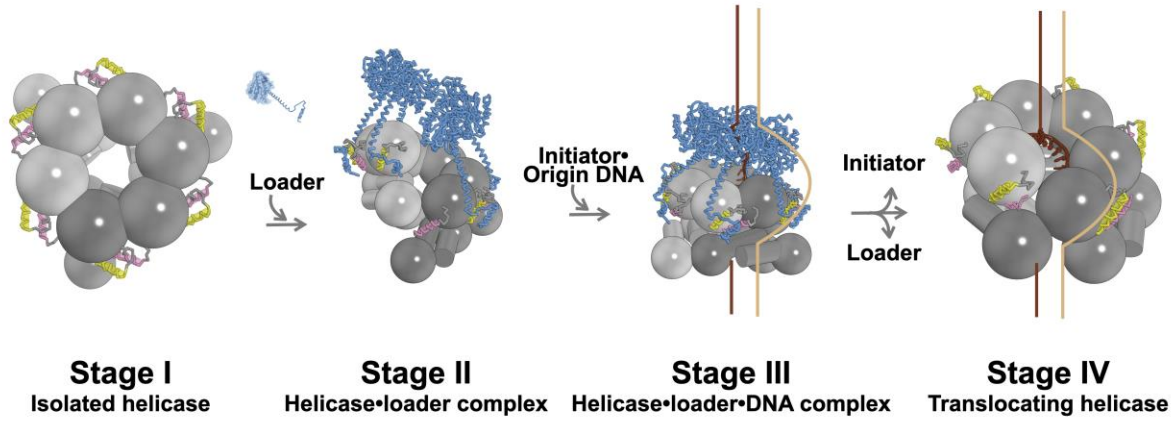


Figure 1. Loading of the Bacterial DnaB Replicative Helicase at a Replication Origin. The DnaB loading pathway passes through at least four stages (I, II, III, and IV). DnaB sub-domains are depicted according to their overall shape (amino-terminal domain (NTD): a mushroom-like shape; carboxy-terminal domain (CTD): sphere; both in varying shades of gray). The Linker-Helix (LH, pink) and Docking-Helix (DH, yellow) elements are depicted in a ribbon and transparent cylinder representation. The DnaC helicase loader is shown as a blue ribbon. The DNA strands, one of which is in included in the central DnaB chamber, and the second excluded, are colored in chocolate brown and light brown, respectively.

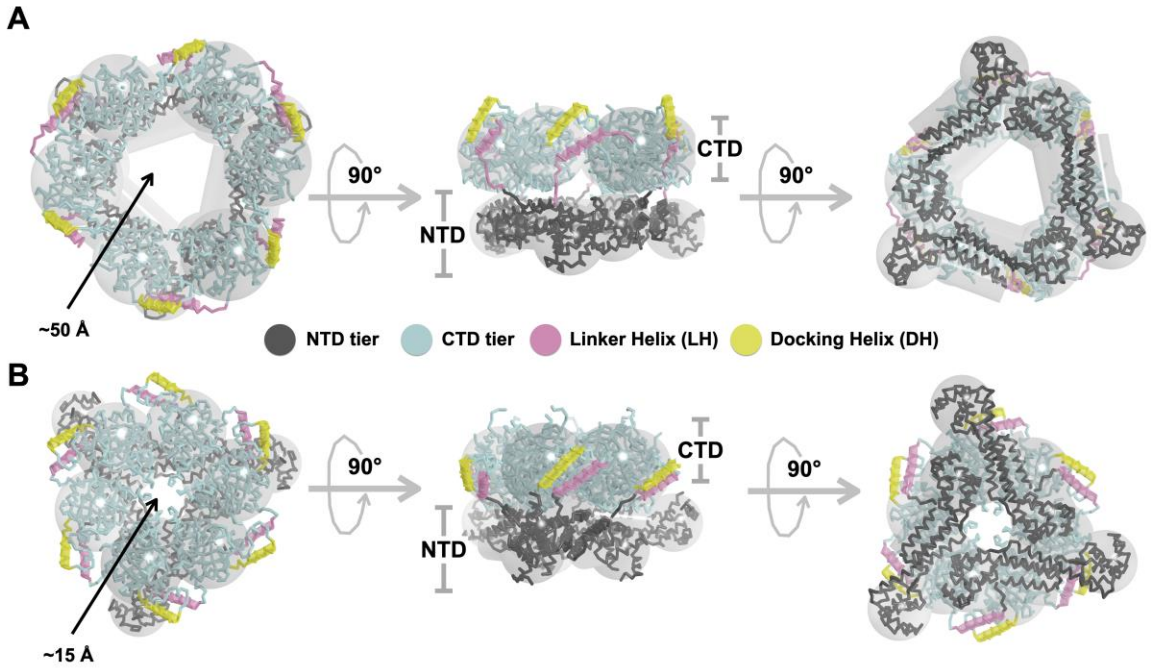
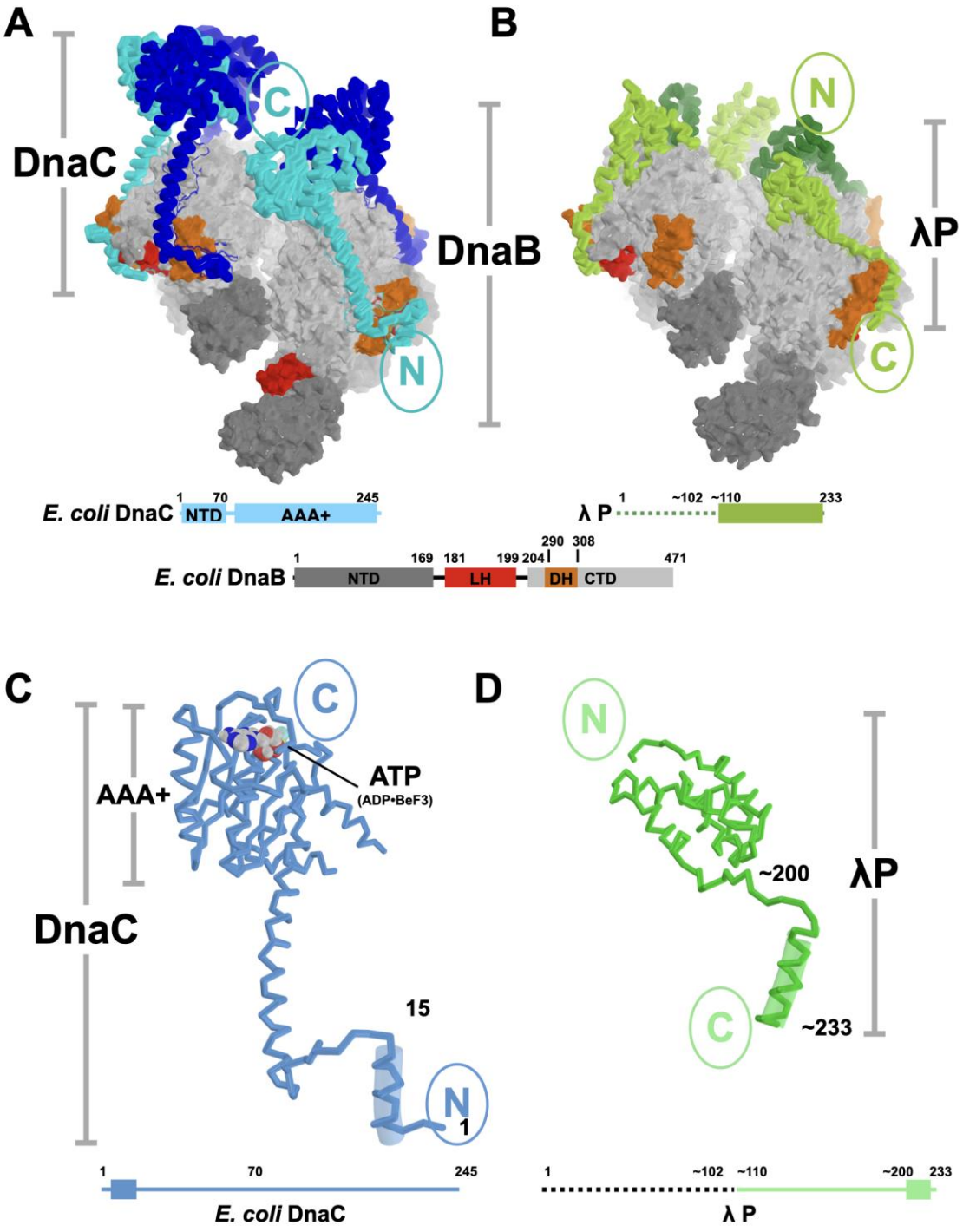


Figure 2. Overview of the DnaB replicative helicase. DnaB adopts at least two distinct configurations, termed dilated (A) and constricted (B) [62–64]**.** DnaB sub-domains are depicted according to their overall shape (amino-terminal domain (NTD): a mushroom-like shape; carboxy-terminal domain (CTD): sphere; both in varying shades of gray). Superimposed on these shapes of DnaB are ribbon representations, colored in gray and light cyan of the NTD and CTD tiers, respectively, in various poses of the dilated (A, PDB = 2R6A) and constricted (B, PDB = 4NMN) forms of DnaB. The linker and docking helices are depicted as cylinders, and colored pink and yellow, respectively.



458
459 **Figure 3. *E. coli* DnaB Complexed with the DnaC (A) and λ P (B) Helicase Loaders.**
460 Protomers of the DnaC and λ P helicase loaders are colored in alternating shades of blue
461 (DnaC) and green (λ P). The *E. coli* DnaB helicase in each loader complex is represented
462 in a surface rendering, with the amino-terminal domain (NTD) and the carboxy-terminal
463 domain (CTD) layers colored in darker and lighter shades of gray, respectively. Linker-

helices (LH) and docking-helices (DH) are in colored in red and orange, respectively. The DnaB hexamers from the loader complexes are superimposed on the CTD of the protomer at the bottom of the spiral in this pose. The primary sequence of each loader and DnaB is represented as a linear schematic, with salient features annotated and colored to match the molecular representations. Only the carboxy terminal domain of λ P was visible in the EM maps of the BP complex (the missing segment is depicted as a dashed line). The terminal helix of each loader (DnaC: N-terminal; λ P: C-terminal) are depicted as ribbons and transparent cylinders. The amino (N) and carboxy (C) termini of each loader is indicated in each panel.

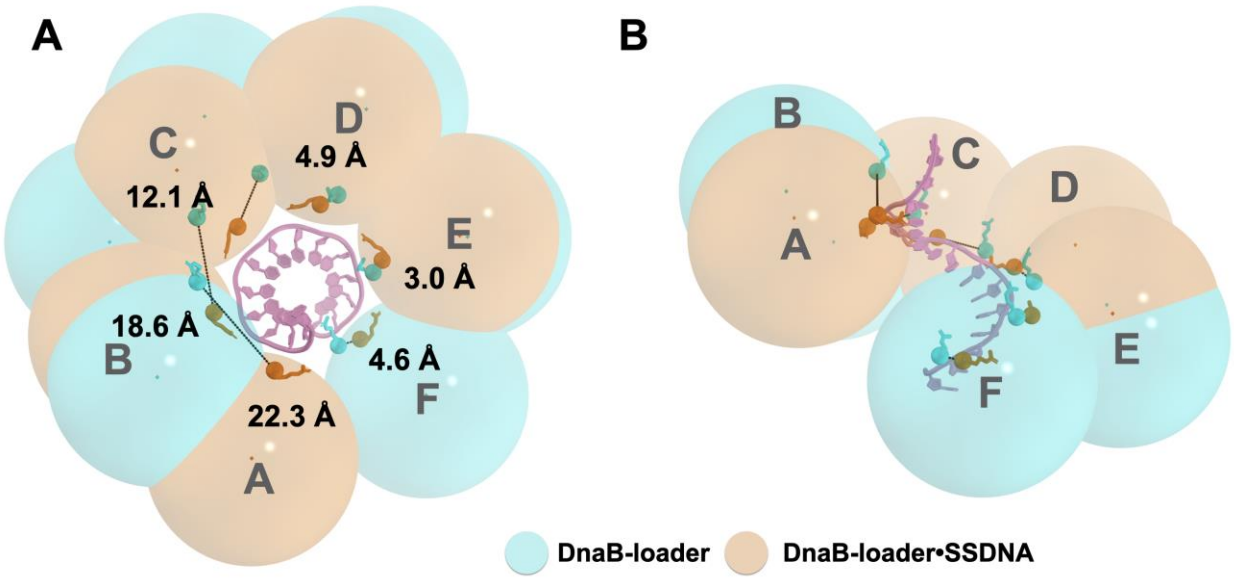


Figure 4. The ssDNA Binding Site is Altered in the Loader Bound Form of DnaB. The BC complex without ssDNA (PDB = 6QEL) was superimposed (RMSD = 0.85 Å) onto the C-terminal domain of chain F of the ssDNA bound complex (PDB = 6QEM). The large spheres represent the CTDs of the BC complex with (light brown) and without (cyan) ssDNA. The alpha carbons of arginine 403, which makes a key contact to ssDNA in the BC complex, are depicted as smaller spheres for the ssDNA complex (brown) and apo complex (dark cyan). Distances between the alpha carbons of arginine 403 from equivalent DnaB subunits are shown. The six chains from the ssDNA-bound complex are identified by letters (A, B, C, etc.). Poses in panels A and B are related by a 90° rotation about the horizontal axis.

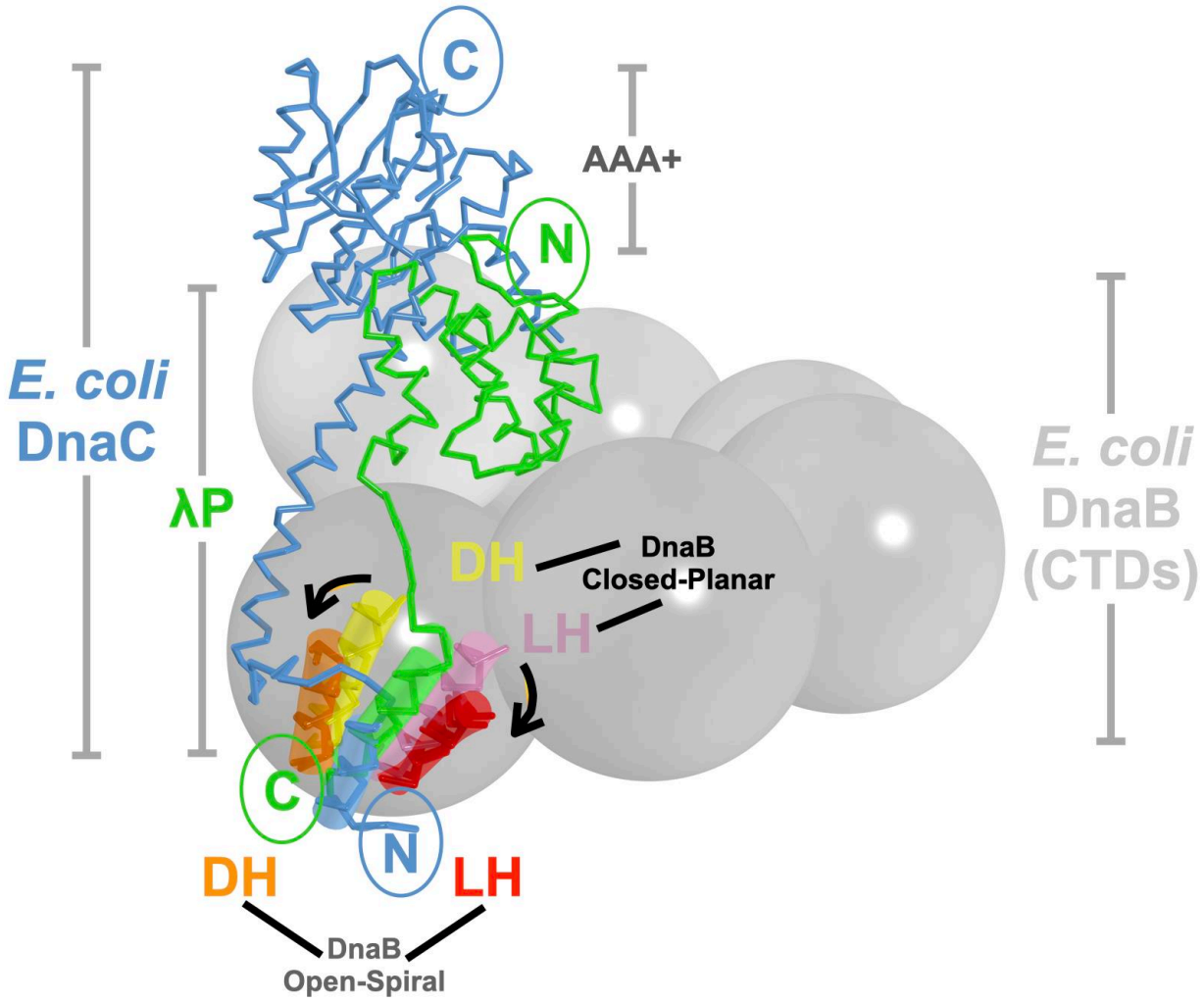


Figure 5. Convergent Evolution/Molecular Mimicry in the Mechanism of Opening of the *E. coli* DnaB Replicative Helicase by the *E. coli* DnaC and λ P Helicase Loaders. The DnaC and λ P helicase loaders are shown in a ribbon representation, colored light blue (DnaC) and green (λ P), with their respective amino and carboxy termini marked. For clarity, only one copy of each loader is shown. The DnaB hexamer from each loader complex is superimposed on the carboxy-terminal domain (CTD) of the protomer at the bottom of the spiral in this pose; the docking helix element was excluded from the alignment to produce an RMSD of 1.3 Å. For clarity, only the CTD tier of DnaB, represented as a set of spheres, colored in varying shades of gray, is shown. The linker (LH) and docking (DH) helices are depicted as a ribbon and transparent cylinder. The LH and DH from the closed-planar form of DnaB are colored yellow and pink, respectively; the corresponding elements from the DnaC and λ P loader-bound DnaB are in orange and red, respectively. The black arrow signifies direction of motion of the LH and DH elements on binding the DnaC and λ P loaders.

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