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Bacterial replisomes

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Abstract
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Bacterial replisomes

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Bacterial replisomes are dynamic multiprotein DNA replication machines that are inherently difficult for structural studies. However, breakthroughs continue to come. The structures of *Escherichia coli* DNA polymerase III (core)–clamp–DNA subcomplexes solved by single-particle cryo-electron microscopy in both polymerization and proofreading modes and the discovery of the stochastic nature of the bacterial replisomes represent notable progress. The structures reveal an intricate interaction network in the polymerase–clamp subassembly, providing insights on how replisomes may work. Meantime, ensemble and single-molecule functional assays and fluorescence microscopy show that the bacterial replisomes can work in a decoupled and uncoordinated way, with polymerases quickly exchanging and both leading-strand and lagging-strand polymerases and the helicase working independently, contradictory to the elegant textbook view of a highly coordinated machine.

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Introduction

Genetic information of living organisms is stored in chromosomal DNA. To faithfully pass it on to the next generation, it is essential that DNA be copied with high efficiency and fidelity. All organisms from bacteria to humans use complex multi-protein molecular machines, called the replisomes, to achieve this feat. Although general functions and mechanisms of replisomes from different domains of life are similar, the components and mechanistic details can be distinct. Here, we focus on bacterial replisomes, particularly that from *Escherichia coli*.

Bacterial DNA replication can be divided into three stages: initiation, elongation and termination. Each stage requires a different set of proteins with highly coordinated activities [1]. The details of each stage as well as recent insights into the structures and functions of protein components or subcomplexes are discussed separately.

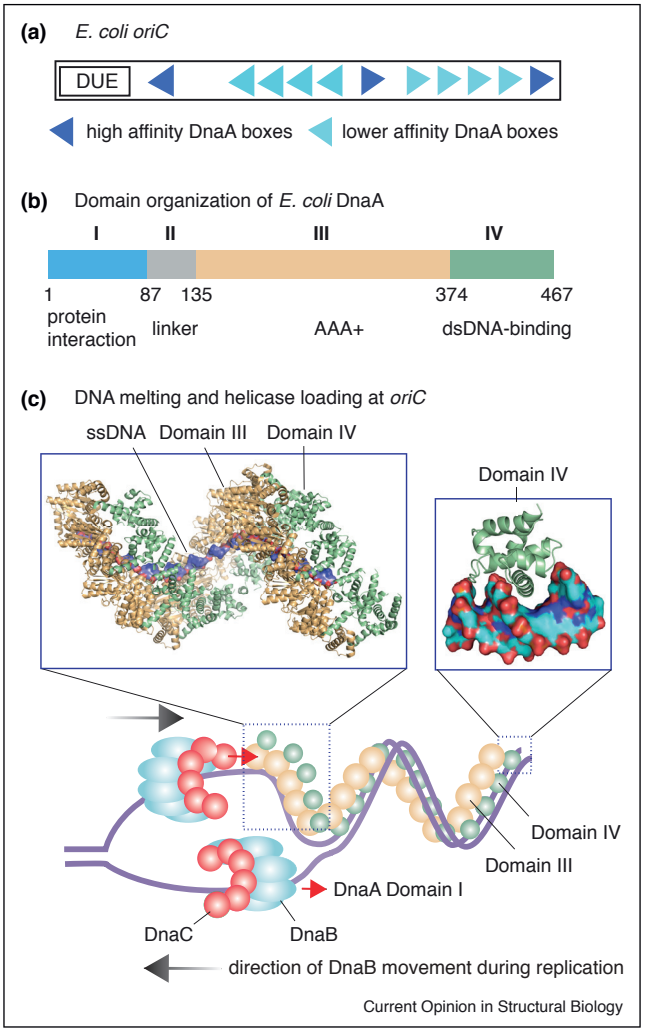
Initiation of DNA replication

Initiation of bacterial DNA replication is tightly controlled to ensure that the chromosome is duplicated once every cell division. Bacterial chromosomes are usually circular doubled-stranded (ds) DNA molecules with a single initiation locus called the replication origin, *oriC*. The *E. coli* chromosome is 4.6 Mb in size with a 250-bp *oriC*. Although there are significant variations in the length and organization of origins in different bacterial species, they are generally comprised of an array of 'DnaA boxes' for origin recognition by the initiator protein DnaA, together with an adjacent AT-rich DNA unwinding element (DUE) for strand separation [2] (Figure 1a). Recently, a string of repeating trinucleotides (5'-TA$^{G}/_{A}$) in the DNA unwinding region, termed DnaA-trio, was identified as an important element [3•].

DnaA has four domains (Figure 1b). The protein interaction domain 1 interacts with protein partners, including the replicative helicase DnaB, and domain 2 is a flexible linker. Domain 3 is the AAA+ ATPase domain, which mediates DnaA oligomerization and binding to single-stranded (ss) DNA [4]. Domain 4 is the dsDNA-recognition domain that binds to DnaA boxes via a helix-turn-helix motif [5] (Figure 1c). Both ATP-bound and ADP-bound DnaA can bind to high-affinity DnaA boxes, but only ATP-DnaA binds to lower affinity boxes and oligomerizes to form a helical filament on *oriC* [6,7] (Figure 1a, c). DNA wrapping around the DnaA filament causes torsional strain in the DUE, contributing to DNA melting [8,9]. The DnaA filament then extends beyond the DnaA boxes with the AAA+ domain interacting with DnaA-trio. This sequesters and stretches one strand of the DUE, facilitating DNA melting and bubble formation for helicase loading [4] (Figure 1c).

After forming a DNA bubble, two DnaB helicase hexamers are recruited and loaded onto each of the separated ssDNA strands as $DnaB_6$–$(DnaC)_6$ complexes. Binding of the helicase loader DnaC inhibits the ATPase and helicase activities of DnaB and traps it like an open right-handed lockwasher, ready to be loaded onto ssDNA [10,11]. DnaC is a homolog of DnaA. Its AAA+ domain interacts with the AAA+ domain of DnaA at the end of the filament and serves as an adaptor to load one DnaB–DnaC

Figure 1



Schematic representation of the initiation of bacterial DNA replication. **(a)** *E. coli oriC*, showing DnaA boxes and the AT-rich DNA unwinding element (DUE). The DnaA boxes contain 9 base pairs with consensus sequence 5'-TTATNCACA (6). The high-affinity DnaA boxes are colored in dark blue and lower affinity boxes in light blue. **(b)** Domain organization of *E. coli* DnaA replication initiator protein. **(c)** DNA melting at *oriC* and loading of the $DnaB_6$–$(DnaC)_6$ helicase–loader complex onto the DNA bubble. Lower schematic: ATP-bound DnaA binds to DnaA-boxes via Domain IV, thereby promoting dsDNA to wrap around the DnaA filament, causing torsional strain to the dsDNA [8,9]. Meantime, Domain III of DnaA binds to one of the two ssDNA strands of DUE and stretches the strand. These interactions cause the AT-rich DUE to melt, forming a bubble [4]. At the same time, binding of DnaC traps DnaB like an open lockwasher, to enable its loading onto ssDNA [10]. DnaC interacts with DnaA at the end of the filament and serves as an adaptor to load one DnaB–DnaC complex [12]. It is not known if closing of DnaB around ssDNA to form a hexameric ring occurs before or concomitantly with dissociation of DnaC. Domain I of DnaA interacts with the N-terminal domain of DnaB, helping to load another DnaB–DnaC on the complementary strand [2]. Upper insets: The helical filament of DnaA formed by Domains III (light orange) and IV (pale green) of *Aquifex aeolicus* DnaA (PDB: 3R8F [4]) and Domain IV of *E. coli* DnaA (pale green) bound to dsDNA (PDB: 1J1V [5]). The ssDNA binds in the middle of the DnaA filament via interactions with the AAA+ Domain III of DnaA.

complex onto the strand that DnaA is stretching [12]. Domain I of DnaA interacts with DnaB of the other DnaB–DnaC complex, helping to load it on the complementary strand [2] (Figure 1c).

In Gram-positive bacteria, such as *Bacillus subtilis*, the hexameric replicative helicase DnaC (counterpart of DnaB) is believed to be assembled from individual subunits with the assistance of the helicase loader DnaI and two others proteins, DnaD and DnaB [6]. In *Helicobacter pylori*, a bacterium with no identified helicase loader, DnaB assembles as a head-to-head double hexamer, which later separates into two hexameric helicases [13].

Next, the DnaG primase interacts with the N-terminal collar of $DnaB_6$, stimulating DnaC dissociation [14]. The two DnaB hexamers later move to the apices of the bubble to form two replication forks moving in opposite directions. DnaG recognizes specific priming sites (preferentially 5'-CTG) to produce a leading-strand RNA primer for DNA elongation, and to repeatedly prime Okazaki-fragment (OF) synthesis on the lagging strand.

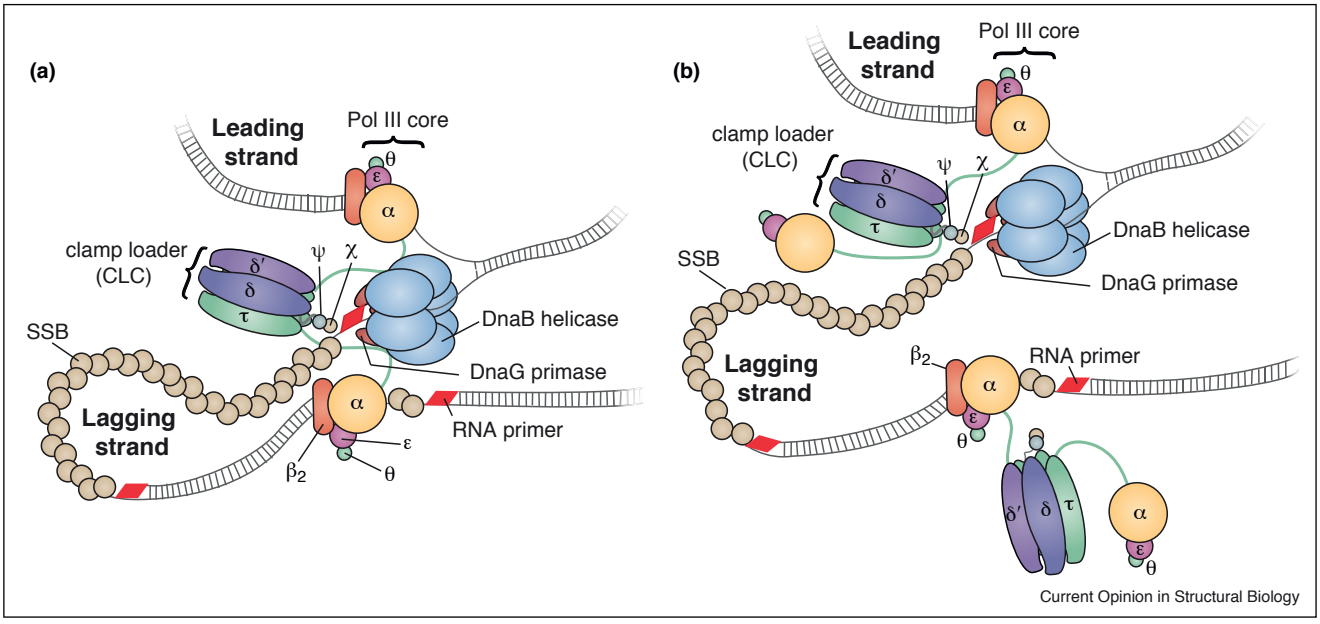
Elongation stage of DNA replication

DNA contains two antiparallel strands that have been thought to be replicated simultaneously by the same replisome. The leading strand is replicated continuously, while the lagging strand is synthesized as short Okazaki fragments. RNA primers of OFs are replaced by DNA by gap filling and nick translation by DNA polymerase I, and the nicks are sealed by DNA ligase.

In *E. coli*, the major replicative polymerase is the Pol III holoenzyme (HE) comprised of 10 different proteins organized into three functionally distinct but physically interconnected assemblies: the $\alpha\varepsilon\theta$ polymerase core, the β_2 sliding clamp and the $\delta\tau_n\gamma_{3-n}\delta'\psi\chi$ clamp loader complex [1] (Figure 2a). In the polymerase core, α is the polymerase subunit, ε the 3'–5' proofreading exonuclease and θ is a small subunit that stabilizes ε. After a RNA primer is made by DnaG, the β_2 clamp is loaded onto the primer terminus by the clamp loader. The α and ε subunits separately bind the clamp, each via a short linear clamp-binding motif (CBM) to the two symmetrically related CBM-binding pockets of β_2. Tethered to the clamp, Pol III is able to synthesize DNA at high speed ($\sim$1000 Nt/s) and with much higher processivity (>150 kb) [1,15].

Bacterial replisomes are highly flexible and mobile machines, their dynamics being mediated and controlled by a network of protein–protein interactions of different strengths. Many of the replication proteins are either conformationally flexible or contain flexible or unstructured regions, making structural studies by X-ray crystallography or NMR difficult. However, through decades of efforts, structures of all *E. coli* replication proteins or their

Figure 2



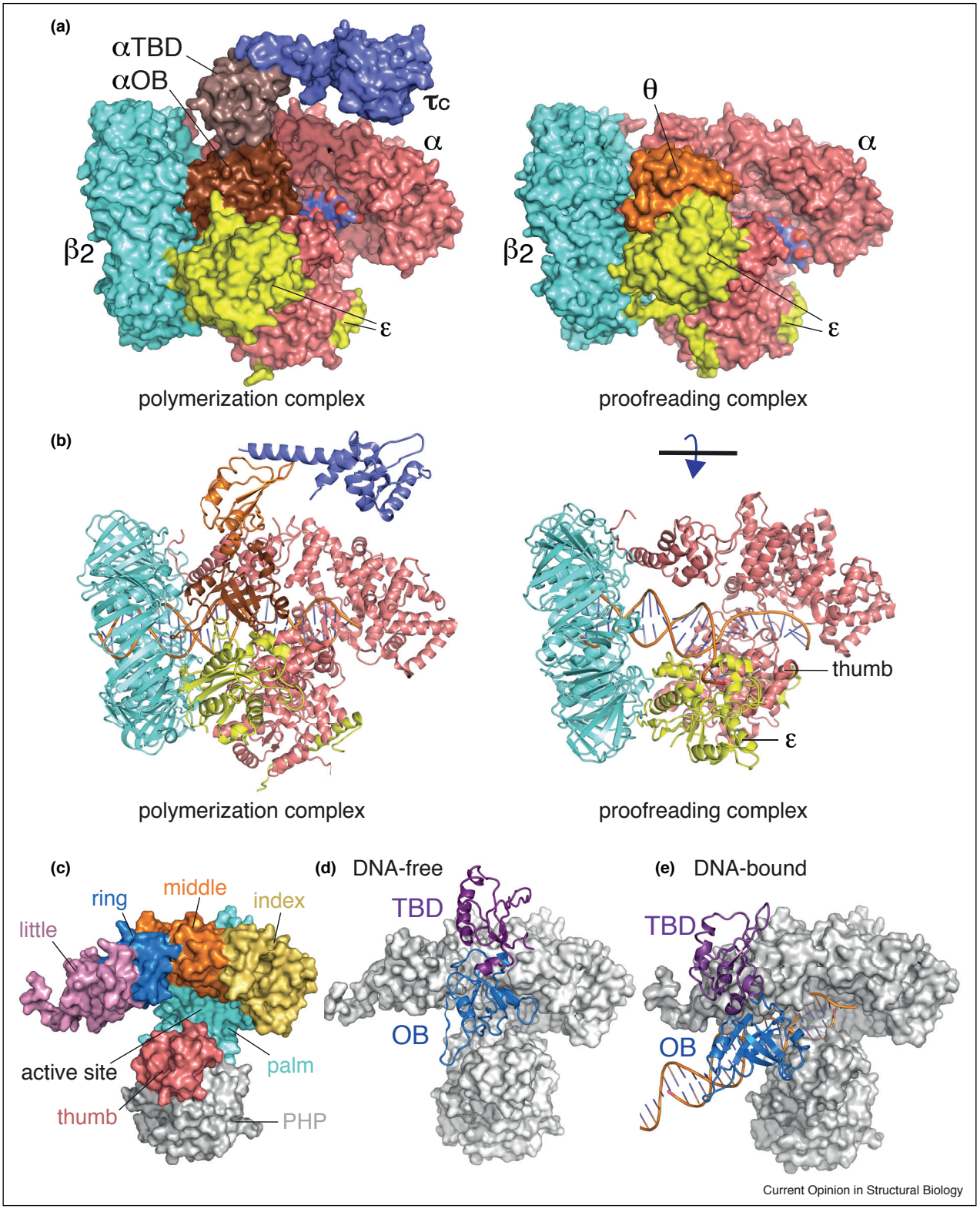
Schematic representation of the *E. coli* replisome adapted from Lewis *et al*. [1]. **(a)** Textbook model of the *E. coli* replisome with coupled and highly coordinated leading-strand and lagging-strand synthesis. Pol III* is connected to DnaB via the τ subunit of the clamp-loader complex and two or three polymerase cores of the same Pol III* replicate both leading-strand and lagging-strand DNA. The ssDNA in the lagging-strand loop is bound by ssDNA-binding protein, SSB. **(b)** Recent studies have shown that *E. coli* Pol III* is readily exchangeable at the fork [33••,34••,35••] and that leading-strand and lagging-strand synthesis may not be tightly coupled, or may even be accomplished by different Pol III HEs. The DnaB helicase can also be decoupled from polymerases and translocate ahead at the apex of the fork [36•].

bacterial homologs have been solved as complexes, whole proteins or domains [1]. Recent breakthroughs in single-particle cryo-electron microscopy (cryo-EM) have seen structures determined of large replisome subassemblies, even the whole bacteriophage T7 replisome, though so far only at modest resolution [16••,17••,18•].

Cryo-EM structures of the *E. coli* Pol III core–clamp–τ_C (C-terminal domain of the τ subunit of the clamp-loader) complexes on primer–template DNA in both polymerization and proofreading modes were recently solved at 8 and 6.7 Å, respectively, along with structures of a DNA-free complex [16••,17••] (Figure 3). These structures resemble previously proposed structural models [15,19,20], with some surprises. For example, in the DNA-bound polymerization complex, the β_2 clamp becomes almost perpendicular to the DNA strands (Figure 3a,b), in contrast to its tilted configuration in the crystal structure of DNA-bound β_2 [21]. While the Pol III α polymerase subunit binds to DNA in a conformation similar to the crystal structure of DNA-bound *Thermus aquaticus* (*Taq*) α, the locations of C-terminal domains (αCTD, comprising the OB and the τ-binding, TBD, domains) are different [22,23]. In the *Taq* α structure and the DNA-free complex, the αCTD is close to the polymerase active site with the OB domain positioned to bind and deliver the ssDNA template into the active site (Figure 3c,d). In the DNA-bound cryo-EM structures, these domains are shifted toward the little finger domain of α, the domain that directly contacts the β_2 clamp; they are therefore far away from the template strand entering the active site (Figure 3e). The OB domain contacts both the little finger and thumb domains of α as well as the β_2 clamp and ε. The face of the OB domain that had been thought to be involved in ssDNA template binding [24,25] now directly faces and is relatively close to the dsDNA. Additionally, ε wedges between the α thumb domain and the clamp. This previously unappreciated interaction network apparently stabilizes the whole complex.

The proofreading complex is fairly similar to the polymerization complex, with small movements of individual protein components [17••] (Figure 3a,b). The most significant movements include a rotation and a tilt of duplex DNA against the plane of β_2, locking the DNA against the inner surface of the β_2 ring (Figure 3b). The polymerase thumb domain and ε also move towards the DNA. The thumb domain wedges between two DNA strands with unmatched base pairs, resulting in a highly distorted and frayed DNA substrate. The newly synthesized strand is therefore able to reach the nuclease active site of ε for editing. Considering that the proofreading complex is fairly similar to the polymerization complexes and duplex

Figure 3



Structures of the *E. coli* polymerase–clamp-τ_C–DNA complexes. **(a)** Surface representations of the polymerization (left) and proofreading (right) complexes [16••,17••]. The N-terminal domains of α (αNTD, residues 1–963, are colored in deep salmon), and the OB (964–1072) and τ-binding domains (TBD, 1173–1160) of αCTD in brown and dark salmon, respectively, ε in yellow, β_2 in aquamarine, θ in orange and τ_C in slate. The polymerization complex does not include θ, and τ_C and the αCTD are missing from the proofreading complex. **(b)** Cartoon representations of

DNA with two unmatched base-pairs tends to fray, it is proposed that ε works passively by waiting for DNA to reach its nuclease active center when a wrong nucleotide is incorporated rather than responding actively to the misincorporation event [17••]. In a complementary single-molecule biophysical study [26], the clamp-bound Pol III core has been shown to be remarkably stable and processive in the proofreading mode in the absence of incoming dNTPs.

A low-resolution (13.8 Å) cryo-EM structure of the whole bacteriophage T7 replisome, a simpler system functionally similar to the bacterial replisomes, has been reported [18•]. In the structure, leading-strand and lagging-strand gp5 polymerases are placed in asymmetric positions and their conformations and interactions with the gp4 helicase–primase protein are also significantly different. The leading-strand polymerase is in a closed conformation, interacting with both helicase and primase domains of gp4 through its finger and exonuclease domains. On the other hand, the lagging-strand polymerase is in an open conformation and interacts exclusively with two other primase domains of adjacent gp4 subunits using a similar region of the exonuclease domain. The two polymerases also interact with each other through the palm domain of the leading-strand polymerase and the finger domain of the lagging-strand polymerase. The structure provides insights into how the two polymerases are organized within the T7 replisome, which may in future be extended to the host bacterial replisomes.

Coordination of leading-strand and lagging-strand synthesis

While structures of bacterial replisomes and their subassemblies continue to be elucidated, shedding light on their flexibility and dynamics, views of how they work are also undergoing paradigm-shifting changes. It was already known that the bacteriophage T7 replisome, which is far simpler to that from *E. coli*, is highly dynamic, with the replicating polymerases quickly exchanging with external polymerases at forks. Perhaps a new polymerase can be used for every OF and more than one polymerase can simultaneously synthesize different OFs [27]. Polymerases may also be left behind to synthesize OFs behind the forks [28•].

Nevertheless, the bacterial replisomes have long been believed to be highly coordinated, highly processive machines capable of copying the whole chromosome without dissociation. Two or three polymerase cores of the same *E. coli* Pol III HE were believed to synthesize both DNA strands, with the lagging strand polymerase repeatedly being recycled for new OF synthesis. Lagging-strand polymerase recycling has been debated to be triggered by various collision or signaling mechanisms in a well-controlled manner [1,29–31]. However, this elegant textbook view has now been challenged [32]. Recent studies find that bacterial polymerases also readily exchange at replication forks and leading-strand and lagging-strand DNA synthesis may not be tightly coupled.

First, Yuan *et al.* [33••] showed that the *E. coli* Pol III α D403E mutant, which can bind to primed DNA but not extend it, can exchange with replicating polymerases. The exchange happens only when the mutant polymerase is attached to a clamp loader containing at least one τ subunit. Core polymerase itself is unable to exchange. Soon polymerase exchange was reported inside *E. coli* cells and in single-molecule *in vitro* assays. Using fluorescence microscopy to track replisome components inside cells, Beattie *et al.* [34••] were able to show that several components of Pol III* (Pol III holoenzyme lacking β_2), including α, ε, τ, δ and χ, all resided at the forks for about 10 s, only long enough for synthesis of a few OFs. Meanwhile, β_2 stayed for 47 s and the DnaB helicase for 15 min. The very similar exchange times of α, ε, τ, δ and χ suggest that it is Pol III* itself rather than individual polymerase components that quickly exchange, while the DnaB helicase in contrast serves as a stable platform for reassembly of replisomes. Using *in vitro* single-molecule assays, Lewis *et al.* [35••] demonstrated that Pol III* exchanges in a concentration-dependent manner; Pol III* is a stable complex that exchanges as a single entity when it is present in excess in solution, but remains bound and highly processive when no spare Pol III* is available. These studies suggest that *E. coli* DNA replication is not as processive as it had been thought, and leading-strand and lagging-strand synthesis is not necessary tightly coupled, considering there is excess of Pol III* in cells. A more recent *in vitro* single-molecule study showed that leading-strand and lagging-strand DNA synthesis by the *E. coli* replisome can indeed be carried out in a decoupled and stochastic way, in which both polymerases and helicase work independently [36•].

(Figure 3 Legend Continued) complexes showing the differences in the primer–template DNA. In the polymerization complex (left), the DNA has B-form structure, while in the proofreading complex, the primer DNA is frayed with the end of the newly synthesized strand in the active center of ε. The proofreading complex is rotated slightly to show DNA in the active center of ε and the θ subunit is omitted for clarity. **(c)** Surface representation of αNTD from the DNA-bound polymerization complex ([16••], PDB: 5FKV), showing the thumb, palm, fingers, and PHP domains. **(d)** Positioning of the αCTD in the DNA-free complexes (PDB: 5FKU). **(e)** Positioning of the αCTD in the DNA-bound polymerization complex (PDB: 5FKV) [16••]. While the OB domain in the DNA-free complex is close to the active site of Pol III α, it is far away in the DNA-bound complex. The OB domain is colored in marine and the TBD in magenta. The αNTD (gray) in the two complexes shows relatively minor changes compared to αCTD.

Considering the exchange of active polymerases at replication forks, perhaps new Pol III* can be utilized to synthesize new OFs at, or even behind, the replication fork, as happens with the T7 replisome [27,28•] (Figure 2b). Excess Pol III* can wait or scan for a new primer and start to synthesize an OF once a new primer is available. This may render unnecessary the various mechanisms proposed to signal polymerase recycling during or at conclusion of OF synthesis. Simultaneous synthesis of more than one OF using different Pol III*s is also possible, so the lagging-strand polymerase does not need to synthesize faster than that making the leading strand.

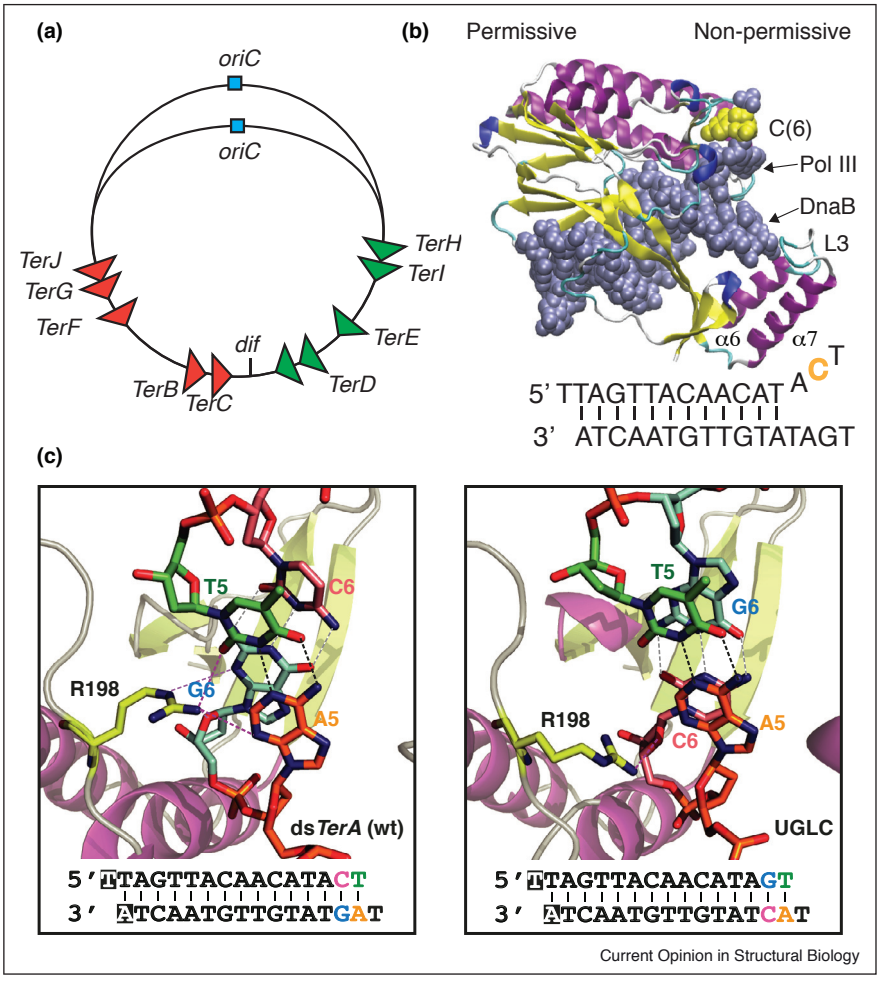
It is instructive briefly to explore how we came to believe in the textbook view of fully coordinated replication by the *E. coli* replisome. After many years of bulk (ensemble) replication assays that defined the importance and roles of the many protein components, it was realized that (initiation) complexes could be assembled on primer–template DNA that could progress, for example on addition of nucleotides, to fast and processive DNA elongation, implying retention of the components of the (initiation) complex within replisomes. Omission of some faster-exchanging components in the elongation stage, like β_2 and DnaG primase, reduced processivity, so these components were routinely added in that stage. The β_2 clamp was subsequently shown also to be capable of recycling from one lagging-strand primer terminus to the next, but to a limited extent, likely governed by whether the clamp-loader complex had already bound a new β_2 clamp from solution [37]. More recent studies, now using single-molecule approaches that reveal alternate pathways for the first time, show that other replisomal components like Pol III* can also exchange when present in excess in solution. Pol III* exchange involves a multipoint competitive interaction mechanism that relies on the hierarchy of strong and weak protein–protein and protein–nucleic acid interactions in the replisome [27,35••,38], and similar mechanisms have now been uncovered in other multiprotein complexes [39–45] and have been mathematically modeled [46–49]. These observations are consistent with the basic principles of chemistry, where multiple pathways can exist in parallel, governed by thermodynamics and kinetics [32]. This redundancy of potential pathways presumably enables timely completion of chromosome duplication in the face of impediments and makes the replisome more resilient to mistakes.

Termination of DNA replication

Proper termination of DNA replication is important for genome stability. *E. coli* replication terminates in the region opposite *oriC*. There are ten 23-bp termination (*Ter*) sites in the region with some sequence variations that determine their binding affinities for the monomeric termination protein Tus [50] (Figure 4a). Tus binds to *Ter* with high affinity in 1:1 ratio, and Tus–*Ter* can further form a very stable 'lock' complex if cytosine-6 of the strictly conserved G–C(6) base pair of *Ter* is flipped out of the DNA duplex and bound in a preformed cytosine-binding pocket of Tus [51] (Figure 4b). The Tus–*Ter* lock complex is polar with a permissive face that allows the replisome to pass unhindered and a non-permissive face that can block the replisome. The ten *Ter* sites are organized as two oppositely orientated groups of five, allowing the replisome to pass the first group and be blocked at the second. This ensures that the two replication forks converge in the terminus region for proper chromosome segregation. However, the blockage efficiency at any single *Ter* site never exceeds 50% *in vivo* [52], a phenomenon that was recently explained. An *in vitro* single-molecule study shows that the proportion of replisomes passing or stalled at a Tus–*Ter* barrier is determined by the speed of the advancing replisome [53••]. Comparison of crystal structures of Tus in complex with different *Ter* variants revealed that the α6/L3/α7 region of Tus undergoes the most significant conformational changes, with residue Arg198 interacting extensively, but differently, with the lagging-strand template before and after lock formation (Figure 4c). It is suggested that competition between the rates of Tus displacement and rearrangement of the Arg198 interaction is critical for lock formation. At high speed, Tus–*Ter* interactions cannot rearrange quickly enough, resulting in Tus dissociation. At lower speeds, the Tus–*Ter* interactions are able to rearrange and the lock forms, permanently blocking the replisome.

Another question concerning Tus–*Ter* is whether specific interactions of Tus with the DnaB helicase are required for replisome blockage. Using magnetic tweezers, Berghuis *et al.* [54•] neatly demonstrated that force-induced, rather than DnaB-induced, separation of duplex DNA is sufficient for Tus–*Ter* lock formation, ruling out the obligate requirement of specific Tus–DnaB interaction for replication fork blockage. The results are consistent with the model that strand separation itself leads to lock formation. This study also identifies three Tus–*Ter* states with different lock dwell times, with the longest-lived state corresponding to the lock and two shorter-lived states likely the intermediates before lock formation [54•,55]. Another study using the T7 replisome showed that the replisome was blocked at the non-permissive face, but T7 polymerase alone proceeds to remove Tus unless the C(6) lock is pre-formed. In contrast, the isolated T7 polymerase approaching from the permissive face is arrested while the replisome and helicase can pass. This suggests that the Tus–*Ter* complex is also sensitive to the translocation polarity of molecular motors, and further argues against the significance of a specific interaction of Tus with DnaB [56•].

Figure 4



Mechanisms of replisome blockage by Tus–*Ter* replication termination complexes. **(a)** Schematic representation of the *E. coli* chromosome, showing positions of *oriC* and *Ter* sites. The clockwise moving fork passes through the permissive sites shown in green and is arrested at the non-permissive sites shown in red. **(b)** Schematic representation of structure of the 'locked' Tus-*Ter* complex (PDB: 2I06), showing cytosine-6 in its binding pocket in Tus. **(c)** Interactions of residue Arg198 of Tus with both strands of *Ter* in complexes with double-stranded wild-type *Ter* (PDB: 2I05, left) and the Tus–*Ter* UGLC complex (GC(6) base pair inverted; PDB: 4XR3, right) [53••].

Conclusions

Bacterial DNA replication and the replisomes that mediate it have been studied extensively for decades. Nevertheless, our understanding continues to develop, and the replisomes are still among the best experimental systems to probe the 'design principles' that determine function of highly dynamic multiprotein machines. Current insights are primarily driven by use of single-particle cryo-EM to probe structures and single-molecule biophysics to probe dynamics. Recent progress includes the cryo-EM structures of *E. coli* polymerase–clamp subassemblies in both polymerization and proofreading modes and the whole phage T7 replisome, coupled with changing views of function driven by single-molecule biochemical studies of the extent of coordination of leading-strand and lagging-strand DNA synthesis by prokaryotic replisomes. Biophysical studies reveal an intricate interaction network in the polymerase core–clamp–clamp loader assemblies, providing functional and structural insights into replisomes. Meantime, ensemble and single-molecule functional assays and fluorescence microscopy show that the bacterial replisomes can work in a decoupled and uncoordinated way, with polymerases able to quickly exchange. Both leading and lagging-polymerases and the replicative helicase appear to be able to work independently, which is contradictory to the textbook view of a highly coordinated machine.

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- • of special interest
- •• of outstanding interest

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The authors report cryo-EM structures of DNA-bound and DNA-free*E. coli* αε–β_2–τ_C complexes at ~8 Å. In both complexes, ε bridges the α thumb domain and the clamp. In the DNA-bound complex, the β_2 clamp rotates and becomes almost perpendicular to the DNA. The polymerase α subunit binds to DNA in a conformation similar to the DNA-bound *Taq* α. However, the C-terminal domains of α undergo significant movements with an ~35° rotation enabling the OB domain to contact the clamp, ε and finger and thumb domains of α, rather than being near the polymerase active site.

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The authors present a cryo-EM structure of the*E. coli* αεθ–β_2–τ_C–DNA complex in the proofreading mode. In the structure, the duplex DNA rotates and tilts against the plane of β_2, locking the DNA against the inner surface of the β_2 ring that stabilizes the complex. The polymerase thumb domain wedges between two DNA strands near the polymerase active center, resulting in a highly distorted and flayed DNA substrate, enabling the newly synthesized strand to reach the nuclease active site of ε for editing. It is proposed that ε works passively by waiting for DNA to reach its active site after an incorrect nucleotide is incorporated.

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This study presents a cryo-EM structure of the whole T7 replisome, in which the leading-strand and lagging-strand polymerases are in asymmetrical positions. Their conformations and interactions with the gp4 helicase–primase protein are different. The leading-strand polymerase is in a closed conformation and interacts with both helicase and primase domains of gp4, while the lagging-strand polymerase is in an open conformation and interacts exclusively with two other primase domains of adjacent gp4 subunits. The two polymerases also interact with each other.

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The authors use multidimensional single-molecule tethered-bead assays to simultaneously image leading-strand and lagging-strand DNA synthesis by the T7 replisome. They observe that most DNA loops form during primer synthesis. The study also suggests that the majority of polymerases loaded onto the new primers are left behind the replisome to complete Okazaki fragment synthesis.

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The *E. coli* Pol III α D430E mutant, which can bind to primed DNA but is incapable of nucleotide incorporation, inhibits DNA synthesis by preassembled replisomes containing wild-type α. The inhibition only occurs when the mutant polymerase core is attached to a clamp loader containing at least one τ subunit. The mutant polymerase core itself does not inhibit, suggesting that Pol III*, and only Pol III*, can exchange with a replicating polymerase.

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The authors study the behavior of replication proteins in *E. coli* live cells using fluorescence microscopy. Their results suggest that Pol III* in the replisome exchanges on a 10 s timescale with free Pol III* from solution. Meanwhile, the DnaB helicase stays bound to the replication fork for much longer, providing a platform for reassembly of a new replisome. Based on the results, it is proposed that both leading-strand and lagging-strand synthesis can be discontinuous.

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Using *in vitro* single-molecule assays with fluorescently labeled polymerases, the authors demonstrate that replicating polymerases can quickly exchange with excess Pol III* complex at replication forks in a concentration-dependent manner. This exchange mechanism ensures a balance between stability and plasticity of the replisome, allowing its components to be replaced when necessary.

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Using single-molecule *in vitro* assays, the authors show that DNA replication by the *E. coli* replisome is kinetically discontinuous. The leading-strand and lagging-strand polymerases function independently and are not coordinated. The DnaB helicase can continue to unwind dsDNA when polymerases pause, but at a significantly reduced rate, allowing polymerases to catch up. The authors suggest that the stochastic behavior of individual components allows DNA to be replicated without coordination of synthesis of both strands.

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This study shows that the proportion of replisome passing or stalled at Tus–*Ter* barriers is determined by the speed at which the replisome is advancing. Comparison with the crystal structures of Tus in complex with *Ter* variants reveals that residue Arg198 of Tus has extensive, but different, interactions with the lagging strand before and after lock formation. It is therefore suggested that competition between rates of Tus displacement and rearrangement of Tus–*Ter* interactions is critical for lock formation.

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The authors use magnetic tweezers to show that formation of a stable Tus–*Ter* lock complex requires physical separation of duplex DNA but not protein–protein interactions, excluding the possibility that a Tus–DnaB interaction is required for replisome blockage. The study also identifies three Tus–*Ter* states with different lock dwell times, with the longest-lived state corresponding to the lock and the two shorter-lived states likely to be intermediates before lock formation.

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This study shows that the T7 replisome is blocked at the non-permissive face of the Tus–*Ter* complex. Surprisingly, isolated T7 polymerase can bypass the Tus–*Ter* barrier from the non-permissive end unless C (6) is unpaired beforehand. In contrast, isolated T7 polymerase approaching from the permissive face is arrested. This suggests that the Tus–*Ter* complex is sensitive to the translocation polarity of molecular motors.