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Review Article

REVIEW ARTICLE

Visualizing mutagenic repair: novel insights into bacterial translesion synthesis

Asha Mary Joseph and Anjana Badrinarayanan*

National Centre for Biological Sciences (Tata Institute of Fundamental Research), Bangalore, Karnataka 560065, India

*Corresponding author: National Centre for Biological Sciences Tata Institute of Fundamental Research Bellary Road, Bangalore 560065, Karnataka, India. Tel: 91 80 23666547; E-mail: anjana@ncbs.res.in

One sentence summary: In this review we highlight the application of microscopy-based approaches to unravel the in vivo mechanism and regulation of bacterial translesion synthesis.

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ABSTRACT

DNA repair is essential for cell survival. In all domains of life, error-prone and error-free repair pathways ensure maintenance of genome integrity under stress. Mutagenic, low-fidelity repair mechanisms help avoid potential lethality associated with unrepaired damage, thus making them important for genome maintenance and, in some cases, the preferred mode of repair. However, cells carefully regulate pathway choice to restrict activity of these pathways to only certain conditions. One such repair mechanism is translesion synthesis (TLS), where a low-fidelity DNA polymerase is employed to synthesize across a lesion. In bacteria, TLS is a potent source of stress-induced mutagenesis, with potential implications in cellular adaptation as well as antibiotic resistance. Extensive genetic and biochemical studies, predominantly in *Escherichia coli*, have established a central role for TLS in bypassing bulky DNA lesions associated with ongoing replication, either at or behind the replication fork. More recently, imaging-based approaches have been applied to understand the molecular mechanisms of TLS and how its function is regulated. Together, these studies have highlighted replication-independent roles for TLS as well. In this review, we discuss the current status of research on bacterial TLS, with emphasis on recent insights gained mostly through microscopy at the single-cell and single-molecule level.

Keywords: bacteria; translesion synthesis; error-prone polymerases; mutagenesis; DNA repair; live-cell imaging; single-molecule microscopy

ABBREVIATIONS

Translesion synthesis:	(TLS)
Nucleotide excision repair:	(NER)
Single-particle tracking:	(spt)
Photoactivable localization microscopy:	(PALM)
Total internal reflection fluorescence microscopy:	(TIRF)

INTRODUCTION

Faithful duplication and segregation of genetic material is fundamental to life. In bacteria, DNA replication initiates at the 'origin' of replication and the replication machinery synthesizes DNA bi-directionally until the 'terminus' where the process is completed (Reyes-Lamothe and Sherratt 2019). Both initiation and termination are highly regulated events. For example, initiation is coupled to nutrient status in some bacteria (for example, via sensing ATP levels), while cell division can be co-regulated with replication termination and chromosome dimer resolution

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(Badrinarayanan, Le and Laub 2015; Leslie *et al.* 2015; Katayama, Kasho and Kawakami 2017; Kleckner *et al.* 2018). Replication is carried out by a multi-subunit machinery called the 'replisome'. A fundamental component of the replisome is the replicative polymerase (Pol III in most bacteria) that faithfully copies DNA with the help of the processivity factor, the β-clamp (McHenry 2011a; Beattie and Reyes-Lamothe 2015; Xu and Dixon 2018). Pol III has a high synthesis rate of ~550–750 nucleotides per second at 37°C in *Escherichia coli* (Pham *et al.* 2013). Additionally, proofreading activity of the polymerase minimizes base misincorporations. Errors that arise despite this are fixed by mismatch repair, thus maintaining a robust check on mutation rates (Kunkel and Erie 2005, 2015; Jiricny 2013), which are estimated to be in the range of 10^{-9}–10^{-10} in optimal growth conditions (Foster *et al.* 2015; Schroeder *et al.* 2018).

DNA damage from endogenous and exogenous sources, including reaction intermediates of biochemical pathways or UV rays (Fu, Calvo and Samson 2012; Ippoliti *et al.* 2012; Fuchs and Fujii 2013), can hamper basic cellular processes including transcription and replication (Cox *et al.* 2000; Cohen, Godoy and Walker 2009; Lang and Merrikh 2018). Under such conditions, replisome progression can be perturbed. For example, studies have observed that mitomycin C-mediated lesions lead to dissociation of the replicative polymerase, PolC, in *Bacillus subtilis* (Li *et al.* 2019), while UV exposure causes delay in replication (Rudolph, Upton and Lloyd 2007) and replisome slowdown in *E. coli* (Soubry, Wang and Reyes-Lamothe 2019). This slowdown is attributed to the inability of Pol III to accommodate bulky DNA modifications and synthesize across them (Yang and Gao 2018). Several outcomes have been proposed for replisome stalling at a lesion: (i) the replicative polymerase is replaced by a low-fidelity polymerase that synthesizes across the lesion, (ii) the replisome skips the lesion, re-primes downstream and continues DNA synthesis or (iii) the replisome disassembles, which can result in a double-strand break (Cox *et al.* 2000; Yeeles and Marians 2011, 2013; Gabbai, Yeeles and Marians 2014). Situations that perturb replication progression lead to accumulation of single-stranded DNA, which could subsequently elicit the SOS response in bacteria (Cohen *et al.* 2008; Baharoglu and Mazel 2014; Marians 2018).

Activation of the SOS response leads to expression of multiple repair-associated genes including various mutagenic DNA polymerases that synthesize across DNA lesions in a process called translesion synthesis (TLS). Unlike the replicative polymerase, TLS polymerases often lack proofreading activity and have structural conformations suitable for accommodating bulky lesions, thus enabling DNA synthesis across them (Napolitano *et al.* 2000; Fuchs and Fujii 2013; Baharoglu and Mazel 2014; Fitzgerald, Hastings and Rosenberg 2017; Yang and Gao 2018). Stress-induced mutagenesis is a potent source for increase in bacterial mutation rates (Fitzgerald, Hastings and Rosenberg 2017; Fitzgerald and Rosenberg 2019) and hence, processes such as TLS are tightly regulated through temporal control of expression of the TLS polymerases and, in some cases, via post-translational modifications of its components specifically in response to damage. In addition, biochemical as well as structural constraints of TLS polymerases limit DNA access and synthesis (Yeiser *et al.* 2002; Vaisman, McDonald and Woodgate 2012; Corzett, Goodman and Finkel 2013; Robinson *et al.* 2015; Jaszczur *et al.* 2016; Vaisman and Woodgate 2017; Yang and Gao 2018; Chang *et al.* 2019). The types and frequency of mutations vary based on the class of TLS polymerase as well as the type of DNA lesion the polymerase bypasses (summarized in Table 1).

The best characterized bacterial TLS polymerases are the B-family polymerase Pol II, and the Y-family polymerases Pol IV (DinB) and Pol V ($UmuD'_2C$) from *E. coli* (Napolitano *et al.* 2000; Goodman 2002; Cohen *et al.* 2008; Sutton 2010; Ippoliti *et al.* 2012; Vaisman, McDonald and Woodgate 2012; Fuchs and Fujii 2013; Goodman and Woodgate 2013; Thrall *et al.* 2017; Henrikus, van Oijen and Robinson 2018). In contrast to *E. coli*, *B. subtilis* utilizes a distinct set of error-prone polymerases: (i) DnaE, the lagging-strand polymerase for primer synthesis, is SOS-inducible and participates in mutagenic repair (Dervyn *et al.* 2001; Le Chatelier *et al.* 2004). (ii) Two Y-family polymerases, YqjH (PolY1) and YqjW (PolY2), contribute specifically to UV-induced mutagenesis (Sung *et al.* 2003). (iii) Additionally, YqjH is also responsible for stationary phase mutagenesis and lagging-strand mutagenesis associated with transcription-replication collisions (Sung *et al.* 2003; Million-Weaver *et al.* 2015; Lang and Merrikh 2018). Several bacteria with GC-rich genomes, such as *Mycobacterium*, *Pseudomonas* and *Caulobacter,* possess two C-family polymerases: constitutively expressed Pol III (DnaE), which performs high-fidelity replication, and damage-induced TLS polymerase, DnaE2, which is responsible for bypass of UV- and MMC-induced lesions (Boshoff *et al.* 2003; Galhardo *et al.* 2005; Warner *et al.* 2010; McHenry 2011b; Lopes-Kulishev *et al.* 2015) (See Table 1).

We refer readers to excellent comprehensive reviews over the last decade for additional information on types, occurrence, substrate preferences and mutational impact of TLS polymerases (Ippoliti *et al.* 2012; Vaisman, McDonald and Woodgate 2012; Fuchs and Fujii 2013; Goodman and Woodgate 2013; Vaisman and Woodgate 2017; Henrikus, van Oijen and Robinson 2018; Yang and Gao 2018). Here, we will discuss the mechanism(s) of TLS and its interplay with replication, with emphasis on insights recently gained from imaging-based approaches (examples of tools used for visualizing these dynamics are provided in Fig. 1 and referred in specific contexts in later sections of this review). Most of these studies have focused their efforts on TLS polymerases in *E. coli*. TLS polymerases in other bacterial systems are less characterized and their *in vivo* regulation and mechanism(s) of action are exciting avenues for future research. We highlight some open questions in this regard in later sections.

MECHANISMS OF TLS REPAIR

Replication-associated TLS

Although replication is stable and processive, replisome components themselves undergo dynamic exchange through the replication process. For example, *in vitro* total internal reflection fluorescence microscopy was used to visualize real-time exchange of fluorescently tagged single polymerase molecules within actively synthesizing individual replisomes of T7 bacteriophage and *E. coli*. These experiments indicated that components of the replication machinery, including the replicative polymerase, are dynamically exchanged with free molecules in solution (Loparo *et al.* 2011; Lewis *et al.* 2017). Real-time tracking of leading-strand DNA synthesis from single replisomes *in vitro* further showed that polymerases exchanged even on the leading-strand during synthesis, contrary to the long-held assumption of continuous synthesis (Graham, Marians and Kowalczykowski 2017). Consistent with these observations, *in vivo* single-particle tracking with photoactivable localization microscopy (spt-PALM) and fluorescence recovery after photobleaching experiments in *E. coli* and *B. subtilis* revealed few seconds dwell times for most replisome components including Pol III (Liao *et al.* 2016; Beattie *et al.* 2017).

Given these results, it is plausible that upon encountering a DNA lesion, the replisome stalls and the replicative polymerase is replaced by a TLS polymerase to synthesize across a lesion

Table 1. Bacterial translesion synthesis repair polymerases.

Polymerase	Lesions	Mutations	Other bacteria studied	References
Pol II B-family (*E. coli*)	G-AAF εC	-2 frameshifts C:G → T:A	*Salmonella typhimurium*	(Paz-Elizur *et al.* 1996; Napolitano *et al.* 2000; Becherel and Fuchs 2001; Al Mamun and Humayun 2006; Koskiniemi and Andersson 2009; Fuchs and Fujii 2013; Goodman and Woodgate 2013)
Pol IV (DinB) Y-family (*E. coli*)	G-AAF ROS NFZ 4-NQ0 BaP MMS	-1 frameshifts A:T → G:C	*S. typhimuriumB. subtilis* (UvrX, YqjH)*C. crescentusMycobacterium smegmatis Mycobacterium tuberculosis Pseudomonas aeruginosa Pseudomonas putida*	(Kim *et al.* 2001; Boshoff *et al.* 2003; Galhardo *et al.* 2005; Jarosz *et al.* 2006; Sanders *et al.* 2006; Bjedov *et al.* 2007; Koskiniemi and Andersson 2009; Hori *et al.* 2010; Kana *et al.* 2010; Williams, Hetrick and Foster 2010; Ippoliti *et al.* 2012; Sharma and Nair 2012; Fuchs and Fujii 2013; Kath *et al.* 2014; Ordonez and Shuman 2014; Million-Weaver *et al.* 2015; Jatsenko *et al.* 2017; Thrall *et al.* 2017; Lang and Merrikh 2018; Johnson, Kottur and Nair 2019)
Pol V (UmuDC) Y-family (*E. coli*)	UV G-AAF ROS	T:A → C:G	*S. typhimuriumB. subtilis* (YqjW)	(Tang *et al.* 2000; Duigou *et al.* 2004; Fujii, Gasser and Fuchs 2004; Neeley *et al.* 2007; Koskiniemi and Andersson 2009; Ippoliti *et al.* 2012; Fuchs and Fujii 2013)
DnaE2 (ImuC) C-family *C. crescentus*	UV MMC	G:C → C:G G:C → A:T	*M. tuberculosisM. smegmatisB. subtilisP. aeruginosaP. putidaMyxococcus xanthus*	(Boshoff *et al.* 2003; Le Chatelier *et al.* 2004; Galhardo *et al.* 2005; Warner *et al.* 2010; Ippoliti *et al.* 2012; Fuchs and Fujii 2013; Lopes-Kulishev *et al.* 2015; Jatsenko *et al.* 2017; Martins-Pinheiro *et al.* 2017; Peng *et al.* 2017)

Abbreviations used in the table: N^2-deoxyguanosine-acetylaminofluorene (G-AAF), N^4-ethenocytosine (εC), reactive oxygen species (ROS), nitrofurazone (NFZ), 4-nitroquinoline *N*-oxide (4-NQO), benzo(a)pyrene (BaP), methyl methanesulfonate (MMS), mitomycin C (MMC).

('TLS at the fork' model via the 'Toolbelt' or 'Mass-action' mechanism) (Fujii and Fuchs 2004; Indiani *et al.* 2009; Kath *et al.* 2014; Zhao, Gleave and Lamers 2017). It is equally possible that the replisome is capable of skipping over the lesion, leaving behind a β-clamp and a single-stranded DNA gap, across which the TLS polymerase synthesizes ('TLS behind the fork' model) (Yeeles and Marians 2011; Marians 2018). In both cases, the β-clamp plays a pivotal role in recruitment of low-fidelity polymerases to the site of a lesion (described in detail below and schematically represented in Fig. 2).

The β-clamp is a central component of the replisome that holds the replicative polymerase in place for DNA synthesis. High processivity of replicative polymerases is mediated by specific interactions with the ring-shaped β-clamp, which encircles and slides on the DNA (Kong *et al.* 1992; Lamers *et al.* 2006; Fernandez-Leiro *et al.* 2015). All TLS polymerases studied so far in bacteria also interact with β-clamp (either directly or indirectly) and this interaction is important for modulating the activity of the polymerase (Wagner *et al.* 2000, 2009; Becherel 2002; Warner *et al.* 2010; Patoli, Winter and Bunting 2013). Indeed, it was recently shown that mutating the β-clamp binding pocket on Pol IV resulted in reduction in both binding events and co-localization with single-stranded binding protein SSB (Thrall *et al.* 2017). β-clamp has two pockets that bind polymerases; one is bound by the replicative polymerase synthesis subunit, α, and the other is bound by the proofreading exonuclease subunit, ε, thus preventing TLS polymerases from accessing the replisome during ongoing replication in steady state conditions (Jergic *et al.* 2013; Toste Rêgo *et al.* 2013). This restriction appears to be bypassed under DNA damage (discussed in detail below).

TLS at the fork

Based on *in vitro* biochemical assays and genetic studies it was proposed that when the replisome stalls at a lesion, a TLS polymerase can replace the replicative polymerase and efficiently synthesize across the lesion in a process referred to as 'TLS at the fork' (Indiani et al. 2005, 2009; Heltzel *et al.* 2012; Kath *et al.* 2014) (Fig. 2A). Given that TLS polymerases including Pol IV and Pol V are induced under DNA damage, initial models suggested that they gain access to the stalled replisome via 'mass-action-based' displacement of the replicative polymerase from the β-clamp. For example, under steady state, there are ~250 copies of Pol IV, which increases ~10-fold during SOS response (Kuban *et al.* 2004); Pol IV overexpression can also result in replication inhibition when induced to high enough levels (Uchida *et al.* 2008). In line with this, multicolor co-localization single-molecule spectroscopy (CoSMoS) experiments showed that Pol III and the TLS polymerase did not bind the β-clamp simultaneously and instead, competed for binding after stochastic dissociation of one of them (Zhao, Gleave and Lamers 2017).

However, as Pol IV is expressed to moderate levels even during normal growth, its access to DNA cannot simply be dictated by laws of 'mass-action'; another layer of regulation is imperative. Consistently, it was observed that mere increase in Pol IV was not sufficient for mutagenesis and that DNA damage was also essential (Moore *et al.* 2017). Furthermore, *in vivo* single-molecule imaging showed that in the absence of damage Pol IV molecules diffused across the entire length of the cell and did not show enrichment along specific cellular positions (Thrall *et al.* 2017). It was also observed that overexpres-

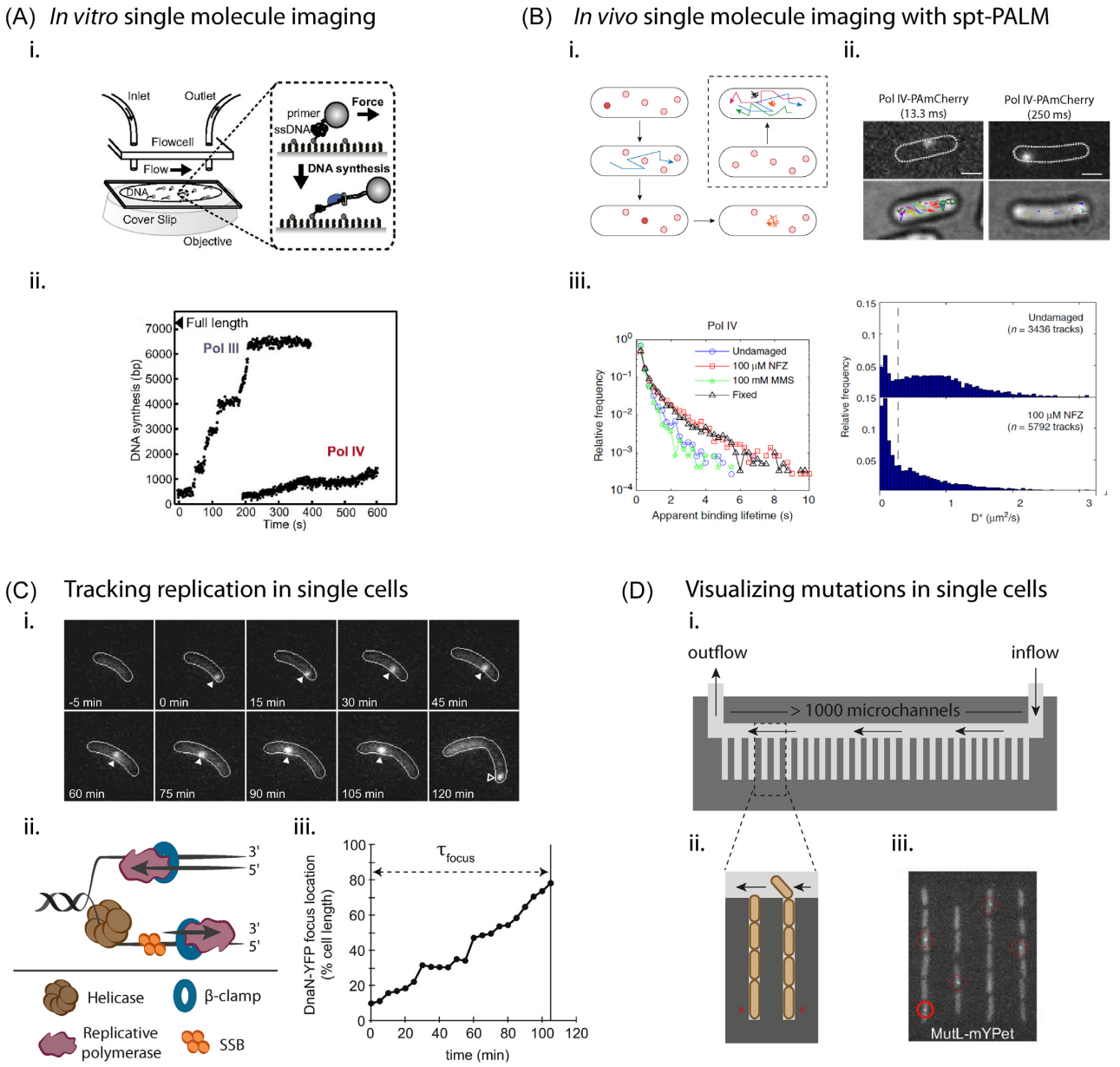

Figure 1. Visualizing repair across scales. **(A)** *In vitro* single-molecule imaging of TLS polymerase activity. (i) Schematic showing the setup used by Kath *et al.* (2014), for visualizing polymerase switching. (ii) Representative trajectories showing Pol IV or Pol III synthesis over time on individual DNA molecules (Kath *et al.* 2014). Images reprinted with permission from PNAS. **(B)** *In vivo* single-molecule imaging of TLS activity. (i) Cartoon representation of the method of single-particle tracking with photoactivable localization microscopy (spt-PALM) (adapted from Stracy *et al.* 2014). Photoconvertible fluorophores are used and only one molecule is activated per cell at any point in time. These molecules are then imaged over time to capture single-molecule trajectories. Populations of freely diffusing, slow diffusing and bound molecules are identified and their characteristics studied. (ii) and (iii) Application of spt-PALM to study Pol IV activity in *E. coli* reveals association of Pol IV with DNA under damage-induced conditions. Images reprinted with permission from Thrall *et al.* (2017) and shared under Creative Commons public license (creativecommons.org/policies). **(C)** Tracking replication in single cells (from Aakre *et al.* 2013). (i) In this example, the β-clamp (DnaN) in *Caulobacter crescentus* is fluorescently tagged with YFP. Replication initiates at one cell pole and is tracked over time until completion at the opposite cell pole, where DnaN dissociates from DNA and its localization is lost. (ii) Schematic representation of the replication fork. (iii) DnaN position over time for cell in (i) is shown. τ_{focus} represents time from focus formation to loss (Aakre *et al.* 2013). Image reproduced with permission from Elsevier. **(D)** Visualizing mutations in single cells. (i) and (ii) Microfluidics devices such as the 'mother-machine' allow the visualization of $>10^5$ cells in a single experiment. This can be combined with fluorescence imaging to follow activity and regulation of repair pathways, such as TLS, in single cells. (iii) This approach has been used to track mutations in real-time by following MutL-YFP foci that denote the position of DNA mismatches (Uphoff 2018). Image reproduced with permission from PNAS.

sion of Pol IV without DNA damage could not result in persistent foci as seen during DNA damage (Henrikus *et al.* 2018). Taken together, another mechanism for 'TLS at the fork' can be considered. A 'Toolbelt' mechanism suggests that replicative and TLS polymerases could be simultaneously bound to the two different polymerase binding pockets on the β-clamp. This can then facilitate fast switching of polymerases to an active DNA synthesis conformation (Indiani et al. 2005, 2009).

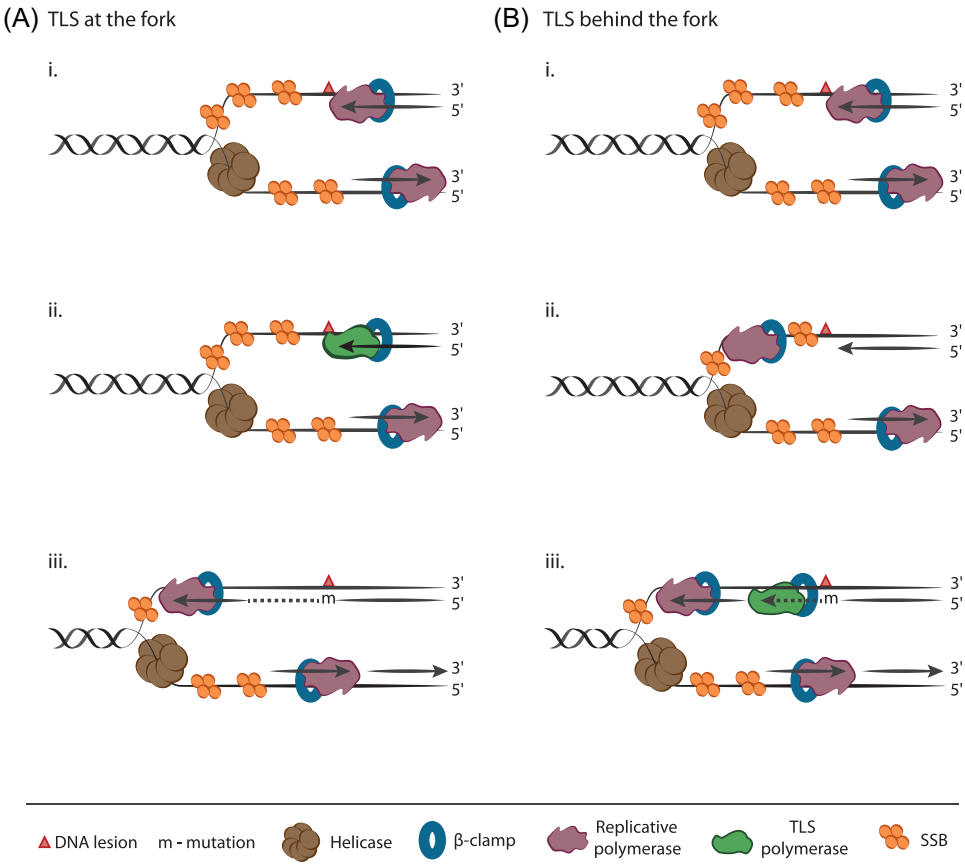

Figure 2. Proposed models for replication-associated TLS. **(A)** TLS at the fork. (i) Replication stalls since the replicative polymerase fails to synthesize past the DNA lesion. (ii) Replicative polymerase switches with TLS polymerase on the β-clamp, while the helicase continues to unwind DNA. (iii) TLS polymerase synthesizes across the lesion albeit with increased probability of incorporating mutations and subsequently the replicative polymerase switches back to continue DNA synthesis. **(B)** TLS behind the fork. (i) Same as in A(i). (ii) Replicative polymerase skips past the lesion, re-primes downstream and continues synthesis, leaving a single-stranded gap behind. (iii) Gap-filling behind the fork is mediated by the error-prone TLS polymerase.

In addition to ensemble biochemical assays, this model was supported by *in vitro* reconstitution and single-molecule imaging of TLS, where synthesis rates of Pol III and Pol IV on individual DNA molecules containing a replication blocking lesion were measured. The switch in synthesis rates when either of the polymerases accessed the DNA provided insights about kinetics of polymerase exchange, which supported a 'toolbelt' model; exchange was faster than recruitment of free molecules from solution and exchange slowed down when the binding pocket for the polymerase on the β-clamp was reduced to one (Kath *et al.* 2014). A detailed study of multiple Pol IV mutants also revealed that interaction of the TLS polymerase with replicative polymerase is required for its function, suggesting that they could be simultaneously bound to the β-clamp (Scotland *et al.* 2015), albeit in a transient manner (Zhao, Gleave and Lamers 2017).

It is important to note though, that constant engagement of an error-prone polymerase with β-clamp can be detrimental if it accesses DNA in an unregulated manner. In support of this idea, a recent study proposed a modified version of the toolbelt mechanism. Consistent with previous studies it was shown that exonuclease subunit (ε) associates with the secondary polymerase binding site on the β-clamp. This binding is critical for the gate-keeping function of ε to regulate promiscuous access of β-clamp by Pol IV (Chang *et al.* 2019). Levels of Pol IV would then dictate whether the TLS polymerase gains access to the replisome at the site of the lesion itself or if the replisome would skip the lesion and TLS would then occur behind the replication fork.

TLS behind the fork

The dynamic nature of the replisome is consistent with the possibility that obstacles can be bypassed by dissociation of the replisome followed by its re-association downstream of the block (Beattie *et al.* 2017) (Fig. 2B). Using an *in vitro* plasmid-based assay, it was previously shown that a DNA lesion on the leading strand only resulted in a temporary stall of replication (Yeeles and Marians 2011). Replication was reinitiated downstream of the lesion, which was dependent on the presence of the primase (DnaG), but independent of replication restart proteins, suggesting that restart is an inherent property of the replisome (Yeeles and Marians 2011, 2013; Yeeles *et al.* 2013). It was further proposed that Pol IV-mediated lesion bypass at the replisome vs lesion skipping by downstream re-initiation could be competing mechanisms (Gabbai, Yeeles and Marians 2014; Marians 2018). This is supported by a recent study that suggests that the gate-keeping function of ε determines the pathway choice between TLS at and behind the fork (Chang *et al.* 2019).

Indeed, several lines of evidence are in favor of the idea that a majority of TLS repair occurs post-replication. It was recently observed that, upon UV exposure, there is an increase in the number of fluorescently tagged Pol III foci that localize away

from active replisomes (denoted by the localization of helicase) (Soubry, Wang and Reyes-Lamothe 2019). Some of these foci could be repair centers performing post-replicative TLS at the lesions that were skipped by active replisomes. This is consistent with observations in methyl methanesulfonate-treated cells, where 61% cells showed an increase in β-clamp localizations (Thrall *et al.* 2017). In another study, time-lapse imaging was performed to follow the dynamics of fluorescently tagged Pol IV during DNA damage by ciprofloxacin, methyl methanesulfonate and UV. It was observed that, under DNA damage, only 5–10% of Pol IV molecules are associated with active centers of replication (tracked using fluorescently labeled τ or ε subunits of the Pol III holoenzyme). The minority of foci that did associate with the replisome showed a broad range of co-localization distances indicating that a significant proportion of these foci could be involved in post-replicative TLS (Henrikus *et al.* 2018; Henrikus, van Oijen and Robinson 2018). Interestingly, Pol IV localization close to active sites of replication dropped dramatically after 90 min of DNA damage, which corresponds to the time when Pol V levels peak, raising the possibility that Pol IV is involved in TLS during early phases, while Pol V takes over this function in later phases of SOS response (Henrikus *et al.* 2018). This is in conjunction with the emerging idea of DNA damage response being chronologically modulated by different sets of proteins and repair pathways that act at discrete time zones of the response (Naiman *et al.* 2014; Fuchs 2016; Fujii, Isogawa and Fuchs 2018; Uphoff 2018).

TLS beyond the replication fork

Taken together, the widely cited model for TLS suggests that it occurs along with replication, either at or behind the fork (Indiani *et al.* 2009; Yeeles and Marians 2011; Heltzel *et al.* 2012; Kath *et al.* 2014; Marians 2018). It is probable that these are competing mechanisms; a combination of both occurs, possibly dictated by the levels of the TLS polymerase at a given instance (Gabbai, Yeeles and Marians 2014; Chang *et al.* 2019). Apart from this, efficiency with which the polymerase can bypass different types of lesions would also have a significant impact on the mechanism of repair (Thrall *et al.* 2017). It becomes important to ask whether these models hold true for other bacteria as well, that encode for different families of TLS polymerases and have distinct rates and mechanisms of DNA replication when compared with *E. coli*.

In contrast to this 'replication-centric' view of TLS, several lines of work (predominantly genetic) highlight a role for this form of repair outside the replisome as well (Fig. 3). A classic, replication-independent function of TLS proposed almost a decade ago is in transcription-coupled TLS (Fig. 3A). If RNA polymerase (RNAP) stalls at a gap on the transcribed strand (most likely generated due to replisome stalling at a lesion on the non-transcribed strand), gap filling mediated by TLS is required to relieve the halted transcription machinery (Cohen, Godoy and Walker 2009; Cohen *et al.* 2010; Cohen and Walker 2011). Additionally, studies have shown that Pol IV and Pol V interact with the transcriptional regulator, NusA (Cohen, Godoy and Walker 2009).

Another interesting role for TLS is resolving collisions between transcription and replication machineries (Million-Weaver *et al.* 2015; Sankar *et al.* 2016; Lang and Merrikh 2018). Transcription and replication machineries traversing from opposite or same directions can crash into each other leading to head-on or co-directional collisions, respectively. Pol IV homolog in *B. subtilis* PolY1 (YqjH) is responsible for increased mutagenesis on the lagging strand in the presence of head-on collisions (Paul *et al.* 2013; Million-Weaver *et al.* 2015). Though head-on collisions are more mutagenic than co-directional ones, the mutation spectra associated with both of them are considerably different suggesting that they occur via distinct mechanistic modes (Srivatsan *et al.* 2010; Sankar *et al.* 2016).

Apart from transcription-associated TLS, homologous recombination is another repair mechanism that has been linked to TLS, and multiple studies show cooperativity between these two pathways (Lovett 2006, 2017; Williams, Hetrick and Foster 2010) leading to mutagenic break repair (Fitzgerald and Rosenberg 2019) (Fig. 3C). It was found that Pol IV can extend D-loops reconstituted *in vitro* and result in -1 frameshifts even in the absence of DNA lesions. Though the exonuclease activity of Pol II suppresses this activity under physiological conditions, Pol IV supersedes Pol II under stress-induced concentrations (Pomerantz *et al.* 2013). In a parallel study, the same group found that Pol I is error prone at RecA-bound recombination intermediates (Pomerantz, Goodman and O'Donnell 2013). Pol IV can also contribute to mutagenic repair of I-Sce1 endonuclease mediated site-specific double-stranded breaks on the *E. coli* chromosome (Shee *et al.* 2011). Consistently, it was observed that fluorescently tagged Pol IV co-localized with RecA at a site-specific double-stranded break in both exponential and stationary phase *E. coli* cells (Mallik *et al.* 2015).

More recently, studies have uncovered cross-talk between TLS and NER (Fig. 3B). Using elegant genetic experiments in combination with mathematical modeling, it was shown that NER and Pol V can act together in processing closely spaced opposing lesions in *E. coli*. Importantly, this type of repair, termed NER-induced mutagenesis, can occur independent of active replication and results in mutations even in stationary phase *E. coli* cells (Janel-Bintz *et al.* 2017). This may be a conserved mechanism of repair across domains of life as coordinated function of NER and TLS has been observed in eukaryotes as well (Kozmin and Jinks-Robertson 2013; Sertic *et al.* 2018).

Finally, in addition to SOS, starvation-induced general stress response also leads to overexpression of TLS polymerases (Shee *et al.* 2011; Fitzgerald, Hastings and Rosenberg 2017). However, overexpression of Pol IV via SOS or general stress responses alone cannot result in mutagenesis. It also requires persistent DNA damage, most likely induced by ROS-mediated oxidative damage (Moore *et al.* 2017). This raises an intriguing possibility of heterogeneity in TLS activity and subsequent mutagenesis, based on the amount of ROS accumulation in individual cells. A recent study has substantiated this by showing increased mutagenesis in a subpopulation of cells with high ROS induction during ciprofloxacin treatment (Pribis *et al.* 2019). Recently developed microscopy assays applied to other repair pathways such as the adaptive response (Uphoff *et al.* 2016) could be used in these contexts to study TLS activity and its regulation in single cells and outside the context of replication.

IMPLICATIONS OF TLS AND OPEN QUESTIONS

In contrast with replicative polymerases that perform high-fidelity synthesis of long stretches of DNA, TLS polymerases perform error-prone, small gap-filling repair synthesis (Fuchs and Fujii 2013; Goodman and Woodgate 2013; Vaisman and Woodgate 2017; Yang and Gao 2018). Low fidelity of polymerases offers the ability to generate genetic variability during DNA synthesis. For TLS polymerases that are induced under stress responses, this feature directly feeds into efficient bypass of a large spectrum of lesions and stress-induced mutagenesis, generating genetic variants that can be resistant to antibiotics or

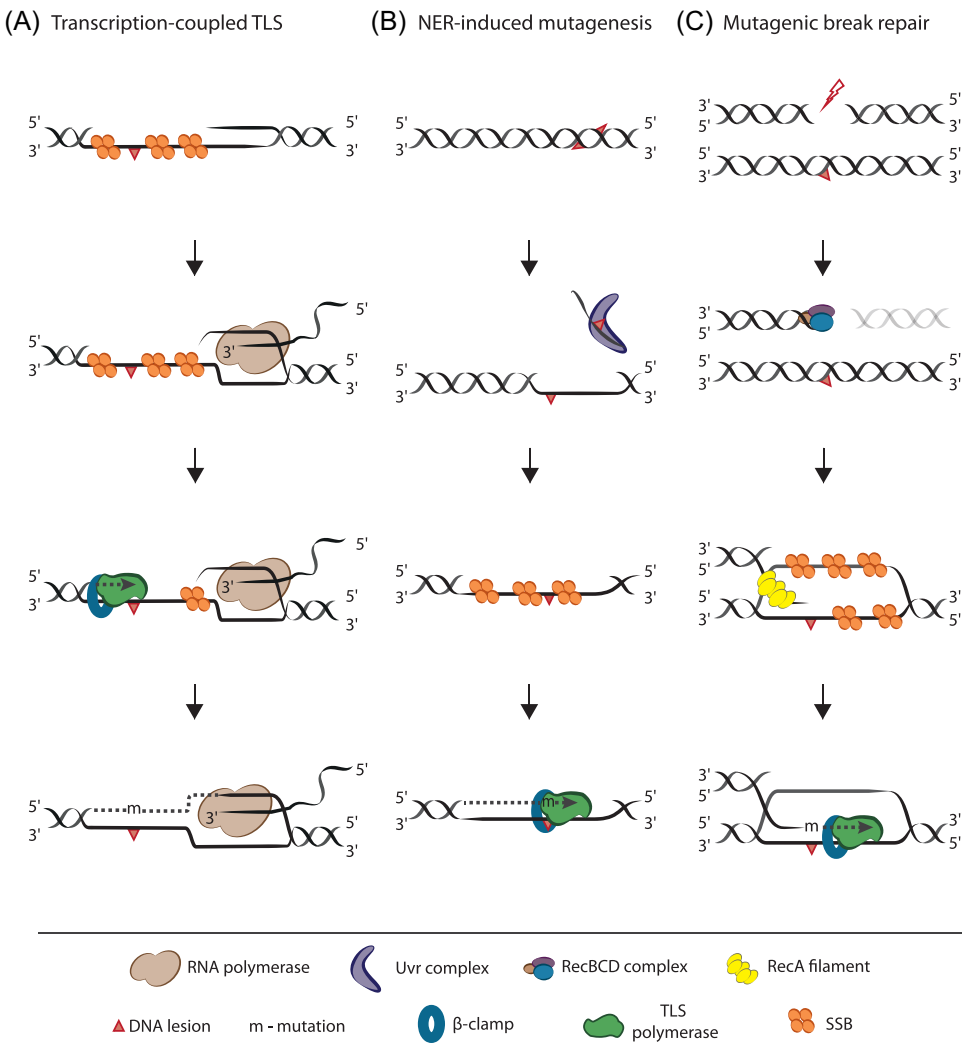

Figure 3. Proposed roles for TLS beyond replication. **(A)** Transcription-coupled TLS. RNA polymerase stalls at a single-stranded gap (likely generated due to perturbation in replication progression) on the transcribed strand. TLS polymerase is then required for gap filling, in the event that a DNA lesion is present on the opposite strand. **(B)** Nucleotide excision repair (NER)-induced mutagenesis. Closely spaced opposing lesions on DNA can result in incision of one of the lesions by NER. Due to persistence of lesion on the opposite strand DNA Pol I fails to fill the gap, thus requiring TLS repair instead. **(C)** Mutagenic break repair. DNA double-strand breaks are processed by the RecBCD (or AddAB) complex to reveal 3′ overhang on which RecA filaments and leads to strand invasion and subsequent repair. In this case, TLS polymerase is required if a lesion is present on the template strand.

better adapted as pathogens (Galhardo *et al.* 2005; Jarosz *et al.* 2006; Warner *et al.* 2010; Foti *et al.* 2012; Robinson *et al.* 2015; Janel-Bintz *et al.* 2017; Moore *et al.* 2017; Cole *et al.* 2018; Henrikus *et al.* 2018; Isogawa *et al.* 2018; Pribis *et al.* 2019).

TLS polymerases are also responsible for certain types of spontaneous mutagenesis in cells such as during replication–transcription conflicts (Million-Weaver *et al.* 2015; Lang and Merrikh 2018). There is ample data to suggest the role of TLS polymerases in stationary phase mutagenesis (Yeiser *et al.* 2002; Sung *et al.* 2003; Corzett, Goodman and Finkel 2013; Janel-Bintz *et al.* 2017; Moore *et al.* 2017). In the past, these assessments have mostly been carried out using fluctuation tests based on forward or reverse genetic approaches, which rely on loss- or gain-of-function mutations of particular selectable markers (Lee *et al.* 2012; Sankar *et al.* 2016; Schroeder *et al.* 2018, Pribis *et al.* 2019) (Table 1). However, these assays are again at the level of population and calculate ensemble averages, ignoring variations at the level of single cells.

In a pioneering approach to track mutagenesis *in vivo*, Elez *et al.* (2010) fluorescently tagged a component of the mismatch repair system (MutL) and visualized its localization in live *E. coli* cells. Persistent MutL foci should bind to mismatches that get converted to mutations in the subsequent generation (Elez *et al.* 2010). Combining this with microfluidics-based approaches, it was possible to simultaneously study mutations by tracking MutL foci in single cells across a significantly large population ($>10^5$ cells) (Uphoff *et al.* 2016; Robert *et al.* 2018; Uphoff 2018). This strategy can be used to understand when and where TLS is active in a population of cells, its propensity for mutagenic or non-mutagenic repair and subsequent impact on cellular survival.

The need to study the error-prone nature of TLS *in vivo* is highlighted by a recent study that revealed the extent of the mutagenic potential of these polymerases. Using deep sequencing techniques, it was shown that Pol V-mediated lesion bypass of a site-specific lesion results in mutations spread across hun-

dreds of nucleotides upstream and downstream of the actual lesion. Interestingly, Pol V creates multiple TLS (mutagenic) patches interspersed with small error-free patches after the lesion, with decreasing mutation frequencies as the patch distance from the lesion increases (Isogawa *et al*. 2018). It is suggested that this mutation patchwork is caused by unique interactions and regulation of Pol V by the 3′ end of RecA nucleoprotein filament, which results in repeated re-association of an otherwise distributive polymerase (Goodman *et al*. 2016; Isogawa *et al*. 2018). It would now be very informative to employ imaging-based tools in this experimental regime to understand the mechanisms by which TLS components are recruited to sites of repair and how their activity may be regulated.

In sum, mechanistic and regulatory investigations in bacteria using imaging-based approaches have been pivotal in understanding the process of TLS. Given the conserved nature of this pathway, these studies have also provided insights into TLS-based repair across domains of life (Fitzgerald and Rosenberg 2019; Xia *et al*. 2019). Most studies have focused their efforts on specific model systems like *E. coli*; in future, it would be important to consider the diversity of TLS polymerases across bacterial species as well (Table 1). For example, *M. smegmatis* has a unique Y-family polymerase Dpo4, which is capable of incorporating rNTPs during DNA synthesis (Ordonez and Shuman 2014; Johnson, Kottur and Nair 2019). Further, presence of distinct families of polymerases (C-family vs Y-family, Table 1) across bacteria highlights the probable role of these polymerases in targeted repair or bypass of lesions that may be faced by cells in their environmental niches or based on their genome GC content. A comprehensive understanding of the specificity, regulation and activity of these polymerases, such as DnaE2 (Zhao *et al*. 2007; Timinskas *et al*. 2014), still remains unexplored, especially at the single-cell and single-molecule level.

In addition, while mechanisms of TLS in the context of replication are well studied, *in vivo* understanding of replication-independent TLS is limited. Studies have only recently begun to focus imaging-based efforts on TLS outside the context of the replisome and/or in response to different DNA lesions. Thrall *et al*. (2017) used spt-PALM to uncover that the type of lesion determined the cellular localization patterns of TLS polymerases. Under methyl methanesulfonate damage, Pol IV molecules were enriched at mid cell, strongly overlapping with localization of single-stranded DNA binding protein, SSB. On the other hand, under nitrofurazone damage, Pol IV molecules were enriched near cell poles (away from SSB). Furthermore, Pol IV localization under nitrofurazone was independent of its β-clamp binding motif and catalytic activity (Thrall *et al*. 2017). Similar two-color imaging of Pol IV with Pol III subunits under various DNA damaging conditions showed that 90% of Pol IV molecules localized away from active sites of replication (Henrikus *et al*. 2018; Henrikus, van Oijen and Robinson 2018). The impact of replication-independent TLS could be significant with respect to mutagenesis in cells devoid of active replication, such as slow-growing bacteria or dormant spores (Janel-Bintz *et al*. 2017). In combination with genetic and biochemical findings, these microscopy-based studies highlight the need to consider the mechanisms and modes of regulation of TLS polymerases that function outside the realms of active replication through cross-talks with other cellular processes and repair pathways.

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