Some supplementary files may need to be viewed online via your Referee Centre at http://mc.manuscriptcentral.com/nar.

Symmetric activity of DNA polymerases at and recruitment of exonuclease ExoR and of PolA to the Bacillus subtilis replication forks

<table>
<thead>
<tr>
<th>Journal</th>
<th>Nucleic Acids Research</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manuscript ID</td>
<td>NAR-03905-J-2018</td>
</tr>
<tr>
<td>Manuscript Type</td>
<td>1 Standard Manuscript</td>
</tr>
<tr>
<td>Key Words</td>
<td>DNA polymerases, DNA repair, Bacillus subtilis, DNA exonuclease, Single molecule microscopy</td>
</tr>
</tbody>
</table>
Symmetric activity of DNA polymerases at and recruitment of exonuclease ExoR and of PolA to the Bacillus subtilis replication forks

Rogelio Hernández-Tamayo¹,², Luis M. Oviedo Bocanegra¹,², Georg Fritz¹,³, and Peter L. Graumann¹,² #.

¹SYNMIKRO, LOEWE Center for Synthetic Microbiology, Marburg, Germany,
²Department of Chemistry, Philipps Universität Marburg, Marburg, Germany,
³Department of Physics, Philipps Universität Marburg, Marburg, Germany.

KEY WORDS: DNA polymerases, Single molecule microscopy, DNA repair, Bacillus subtilis, DNA exonuclease

RUNNING TITLE: Dynamics of DNA polymerases at the single molecule level

ADDRESS FOR CORRESPONDENCE:

#Prof. Dr. Peter L. Graumann
SYNMIKRO, LOEWE Center for Synthetic Microbiology and Department of Chemistry, Philipps Universität Marburg, Marburg, Germany, Hans-Meerwein-Straße 6, 35032 Marburg, Germany. Phone number: +49-6421 28 22210
peter.graumann@synmikro.uni-marburg.de
ABSTRACT
DNA replication forks are intrinsically asymmetric and may arrest during the cell cycle upon encountering modifications in the DNA. We have studied real time dynamics of three DNA polymerases and an exonuclease at a single molecule level in the bacterium Bacillus subtilis. PolC and DnaE work in a symmetric manner and show similar dwell times. After addition of DNA damage, their static fractions and dwell times decrease, in agreement with increased reestablishment of replication forks. Only about 20% of replication forks show a loss of active polymerases, indicating highly robust activity during DNA repair. Conversely, PolA, homolog of polymerase I, and exonuclease ExoR are rarely present at forks during unperturbed replication, but are recruited to replications forks after induction of DNA damage. Protein dynamics are altered, especially during DNA repair, of PolA or ExoR in the absence of each other, indicating somewhat overlapping functions. Purified ExoR displayed exonuclease activity and preferentially bond to DNA having 5’ overhangs in vitro. Our analyses support the idea that two replicative DNA polymerases work together hand in hand at the lagging strand while only PolC acts at the leading strand, and that PolA and ExoR perform inducible functions at replication forks during DNA repair.
INTRODUCTION

DNA replication is carried out in all organisms by a multiprotein complex called the replisome (1-3). Owing to the high level of functional similarity of replication proteins in different species from bacteria and phages to eukaryotes, bacterial replication has been used as a model system (2,4,5). According to textbooks, DNA replication is driven by two or three major DNA polymerases in all cells (6). In eukaryotes, two different polymerases mediate replication at leading or lagging strand, and polymerase I removes RNA primers at the lagging strand (5). In Escherichia coli cells, the same polymerase (PolC) acts at both strands, and polymerase I (PolA) removes RNA hybrids (2,7). The stoichiometry and architecture of the replicative DNA polymerase holoenzyme (Pol III) have been well characterized in the model organism E. coli (8). Although E. coli has served as a prototype for understanding DNA synthesis in vivo, and although some E. coli DNA replication features are conserved across species, the replisomes of many other bacterial species have a distinct organization and may operate differently (9).

The replication machineries in the Gram-positive model bacterium Bacillus subtilis, in Streptococcus pyogenes and in Staphylococcus aureus have also provided useful model systems for understanding unique aspects of DNA replication, from both a genetic and biochemical perspective (10-12). Some features of E. coli replication are conserved (1), however, genetic studies have demonstrated that both PolC and DnaE are required for B. subtilis replication (13). In the absence of DnaE, leading strand DNA synthesis remains active while lagging strand synthesis ceases (14), suggesting that PolC is the leading strand replicase and DnaE the counterpart at the lagging strand (6). Indeed, only DnaE is able to extend primase-generated RNA primers at the lagging strand (1,15,16). However, recent in vitro experiments indicated that PolC also acts at the lagging strand, possibly by extending stretches synthesized by DnaE, which has relatively slow polymerase activity in vitro (1,14), and would not be able to keep up with polymerization speed set by the leading strand. Interestingly, DnaE lacks proof reading activity, and is able to bypass DNA lesions in an error prone
manner (17). Thus, an alternative scenario could be that PolC takes over a proofreading function at the lagging strand.

Structural characterization and biochemical studies of several prokaryotic DNA polymerase I (Pol I, or PolA) enzymes has established an organization into three functional domains: an N-terminal domain associated with a 5′-3′ exonuclease activity, a central domain that mediates proofreading of the 3′-5′ exonuclease activity, and a C-terminal domain responsible for the polymerase activity (18). PolC also contains both DNA polymerase and proofreading 3′→5′ exonuclease activities in one polypeptide chain, whereas DnaE has no proofreading activity (19). Although short DNA fragments containing RNA accumulate in B. subtilis cells in which PolA has been inactivated (20), indicating that it removes RNA primers at the lagging strand similar to Pol I in E. coli, the polA gene is not essential, suggesting that a protein other than PolA performs the essential function of primer removal.

In spite of good knowledge on biochemical properties of the B. subtilis replicative DNA polymerases, the in vivo composition and architecture of the replisome is little investigated. Interestingly, genes encoding proteins acting on the lagging strand have evolved at a significantly higher rate than those dealing with the leading strand (13,14). All these features raise the question whether there is a division of labour between the two replicative DNA polymerases, each one being devoted to one strand (21), or if the B. subtilis replisome is more eukaryotic-like in that it relies on a two DNA polymerase system for chromosomal replication (5), using two polymerases at both strands.

DNA polymerases also play a key role in various DNA repair mechanisms, ensuring faithful chromosome replication and maintenance of their genome integrity (22). This includes the repair of oxidized, alkylated, or deaminated DNA bases, as well as of DNA crosslinks and UV light-induced DNA damage (23). This variety of DNA lesions requires modular repair pathways that carry out damage recognition, damage removal, repair synthesis, and ligation in sequential steps catalysed by a series of enzymes (24,25). Additionally, all repair pathway steps need to be precisely balanced to avoid accumulation of DNA intermediates that are typically more mutagenic
and toxic than the original lesion (26). Rapid processing of gapped and nicked intermediates is particularly crucial (4) because they provoke lethal double-strand breaks upon encountering replication forks (27); a single such break can lead to chromosome loss and cell death. Base-excision and nucleotide-excision repair remove short sections of the damaged DNA, leaving single-stranded DNA gaps to be filled and sealed by Pol I. Indeed, single-molecule tracking experiments revealed transient binding of individual Pol I and ligase molecules in the presence of DNA methylation damage, allowing base-excision repair rates to be quantified in live E. coli cells (28).

Given that the less well-understood B. subtilis replisome appears more eukaryotic-like than the E. coli replisome but appears to be distinct from both systems, a deeper in vivo understanding of how DNA polymerase dynamics occur in B. subtilis is necessary. To quantitatively image how the replicative DNA polymerases move, bind and unbind to the replication machinery in B. subtilis, and also to address the question how they deal with induced DNA damage, we applied single-molecule tracking (8,28,29) to examine the in vivo behaviour of PolC, DnaE, and PolA. We also studied YpcP, which we term ExoR hereafter, which is homologous to the exonuclease domain of PolA. In contrast to E. coli Pol I, PolA is not essential in B. subtilis, which has been surprising given its evolutionary conservation. Interestingly PolA becomes essential in the absence of ExoR (25,30,31), indicating that ExoR might be able to take over an essential function of PolA; whether this occurs at the replication forks is not known.

Reporting on the dynamics of PolC, DnaE, PolA and ExoR proteins within live cells and within regard to the replication machinery, we provide in vivo evidence that B. subtilis replication forks present unusual features not known from replication machineries in E. coli and eukaryotic systems.
MATERIALS AND METHODS

Bacterial Strains and Growth Conditions.

The bacterial strains and plasmids used in this study are listed in Table S1, and the nucleotides are listed in Table S2. Escherichia coli strain XL1-Blue (Stratagene) was used for the construction and propagation of plasmids and E. coli strain BL21 Star DE3 (Invitrogen) for the heterologous overexpression of proteins. All Bacillus subtilis strains were derived from the wild-type strain BG214. Cells were grown in Luria-Bertani (LB) rich medium at 37°C or 30°C or in minimal medium containing S750 salts at 30°C (32). When needed, antibiotics were added at the following concentrations (in µg/ml): ampicillin, 100; chloramphenicol, 5; spectinomycin, 100; kanamycin, 30. When required, media containing mitomycin C (MMC) were prepared by adding appropriate volumes of a filter-sterilized solution 50 ng/ml.

Construction of strains

PolC, DnaE, PolA, ExoR and DnaX were visualized as a PolC-mV, DnaE-mV, PolA-mV, ExoR-mV and DnaX-mV fusion proteins expressed at the original locus. The last 500 bp coding for each gene were integrated into vector pSG1164-mVenus (33), using ApaI and EcoRI restriction sites, and BG214 cells were transformed with this construct, selecting for cm resistance (leading to strains Table S1). For colocalization studies, DnaX-CFP was integrated at amyE locus (by the use of the plasmid pSG1192 (34) and expression was controlled by xylose addition. To investigate colocalization of PolC, DnaE, PolA and ExoR, the resulting strains PG3306, PG3307, PG3308 and PG3309 (see Table S1) was transformed with chromosomal DNA of strains leading expressing PolC-mV, DnaE-mV, PolA-mV and ExoR-mV to DnaX-CFP. A strain with mV-tagged PolA and ExoR in an polA or exoR deletion background strains was generated by transformation of competent ΔypcP::kan trpC2 and ΔpolA::kan trpC2 {obtained from the Bacillus Genetic Stock Center (Columbus, Ohio) (35)} cells with chromosomal DNA from strain expressing either PolA-mV or ExoR-mV.

For expression of soluble 6xHis-ExoR, the coding sequence lacking the first 10 codons was amplified by PCR using chromosomal DNA from B.
subtilis wild type strain BG214. The fragment was further integrated in the expression vector pET28a (Novagen) by EcoRI and XhoI restriction ligation and brought into the expression host E. coli BL21 (DE3) giving rise to the strain pET28a::exoRHisTag.

Protein purification.
Protein purification was performed in two consecutive steps. The purification of (His)6-ExoR initially began with affinity chromatography using an ÄKTA Prime apparatus (GE Healthcare) and Nickel-Sepharose columns (HisTrap HP 1 ml, GE Healthcare) and was continued by size-exclusion chromatography using an ÄKTA FPLC apparatus (GE Healthcare) and a gel filtration column (Superdex 75 16/60 GL, GE Healthcare). For details, see supplementary material.

Fluorescence Microscopy.
For fluorescence microscopy, B. subtilis cells were grown in S750 minimal medium at 30°C under shaking conditions until exponential growth. Conventional light microscopy was performed using a Zeiss Observer Z1 (Carl Zeiss) with an oil immersion objective (100 x magnification, 1.45 numerical aperture, alpha Plan-FLUAR, Carl Zeiss) and a CCD camera (CoolSNAP EZ, Photometrics). Data were processed using Metamorph 7.5.5.0 software (Molecular Devices, Sunnyvale, CA, USA).

Single molecule microscopy and tracking.
In contrast to the wide-field illumination used in conventional epifluorescence microscopy, the excitation laser beam used in our setup is directed to underfill the back aperture of the objective lens, generating a concentrated parallel illumination profile at the level of the sample, leading to a strong excitation followed by rapid bleaching of the fluorophores. When only a few unbleached molecules are present, their movement can be tracked. In addition, freshly synthesized and folded fluorophores become visible when the sample is excited again. When an observed molecule is bleached in a single step during the imaging, it is assumed to be a single molecule (8,36). Image acquisition
was done continuously during laser excitation with the electron-multiplying
CCD (EMCCD) camera iXon Ultra (Andor Technology, Belfast, UK). A total of
1,500 frames were taken per movie, with an exposure time of 20 ms (23
frames per second [fps]). The microscope used in the process was an
Olympus IX71, with a ×100 objective (UAPON 100×OTIRF; numerical
aperture [NA], 1.49; oil immersion). A 514-nm laser diode was used as
excitation source, and the band corresponding to the fluorophore was filtered
out. Of note, cells continued to grow after imaging, showing that there is little
to no photodamage during imaging, while cells stop growing when exposed to
blue light (below 480 nm). Acquired streams were loaded into Fiji ImageJ (37).

Automated tracking of single molecules was done using the ImageJ plugin
MtrackJ, or u-track 2.1.3 (38).

Diffusion analysis of single-molecule tracks.

Tracking analysis was done with u-track-2.1.3, which was specifically written
for Matlab (MathWorks, Natick, MA, USA). Only trajectories consisting of a
minimum of 5 frames were considered tracks and included for further
analysis. A widely accepted method to analyse the diffusive behaviour of
molecules is by using the mean squared displacement (MSD)-versus-time-lag
curve (39,40). This provides an estimate of the diffusion coefficient as well as
of the kind of motion, e.g., diffusive, subdiffusive or directed. However, the
method requires that within a complete trajectory there be only one type of
homogeneous motion and that the trajectory is preferably of infinite length. To
distinguish immobile and mobile molecules from each other, we compare the
frame-to-frame displacement of all molecules in x and the y directions. Using
a Gaussian mixture model to fit the probability density distribution function of
all frame-to-frame displacements, determine the standard deviations $\sigma_1$ and
$\sigma_2$, as well as the percentages $F_1$ and $F_2$ of the slow and the fast subfractions
of molecules, respectively. Finally, the diffusion constants were calculated
according $D_i = \frac{\sigma_i^2}{2\Delta t}$, $(i = 1,2)$ where $\Delta t$ is the time interval between
subsequent imaging frames (29,40). Additionally, an apparent diffusion
analyses have been carried out (41), applying a maximum likelihood estimation fit to the distribution of the diffusion coefficient of every trajectory. Generation of heat maps, analyses of molecule dwell times, and visualization of slow and fast tracks in a standardized cell are based on a custom-written Matlab script (SMTracker) that is available on request (42). SMTracker can use particle tracking tools u-track (38) and TrackMate (43) and computes the x- and y-coordinates of molecular trajectories relative to the geometry of each cell, as obtained by the cell segmentation tools MicrobeTracker (39) or Oufi (44).
RESULTS

PolA and ExoR do not visibly assemble at the replication forks during exponential growth, but after induction of DNA damage.

Cell biological studies have shown that *B. subtilis* DNA polymerases do not move through the cytoplasm while actively replicating DNA, in contrast to the *E. coli* replisome (45). Rather, the *B. subtilis* replisome has been shown to reside in a more restricted location through which template DNA is pulled in, and newly synthesized DNA is extruded towards the cell poles, which has been suggested to facilitate chromosome segregation (46). Therefore, the position of replication forks can be determined using epifluorescence microscopy and fluorescent protein fusions to components of the machinery.

In order to address the question whether PolA and ExoR are also components of the replisome, we generated mVenus (mV) fusions to the C-terminus of ExoR and to the three DNA polymerases PolA, PolC and DnaE, which were integrated at the original gene locus on the chromosome. Thereby, solely fusion proteins are expressed under the control of the original promoter, ensuring the expression of wild type levels for all proteins (Table S1). Presence of full-length proteins (and no free mVenus or degradation products) was verified using Western blotting (Fig. S1). PolC-mV and DnaE-mV fully complemented the wild type copies, while PolA-mV and ExoR-mV showed slightly reduced survival in response to treatment with 40 µg/ml mitomycin C (MMC), but not to 50 ng/ml (Fig. S2A). Because PolA-mV and ExoR-mV expressing cells were much more viable than the corresponding mutant cells (Fig. S2), both fusion proteins retain almost full functionality compared to the wild type proteins.

Epifluorescence experiments showed that while PolC-mV and DnaE-mV colocalized with DnaX-CFP, a component of the clamp-loader complex, and thus visibly accumulated at the forks, neither PolA-mV nor ExoR-mV provided detectable foci during exponential growth (Fig. 1). In order to find out if either of the proteins might be visible at forks in the absence of the other, we moved the FP fusions into the corresponding deletion background. Neither of the two proteins showed foci associated with DnaX-CFP but were visually localized
throughout the cells (Fig. 1), showing that there is no obvious visual complementation.

We used chemical (mitomycin C, MMC) and physical (UV irradiation) damage to assess if a) the DNA III-type polymerases PolC and DnaE remain bound to forks during DNA damage repair, and if b) PolA or ExoR might be recruited to the forks under these conditions. Fig. 2 and Fig. S3 show that replication forks were maintained, as judged by the continued presence of single or two fluorescent DnaX-CFP foci per cells, and forks retained both, PolC and DnaE. Quantification of foci showed that 100 DnaX-CFP foci colocalized with 96 PolC-mV or 92 DnaE-mV foci after MMC treatment, showing that in spite of running into DNA interstrand crosslinks, or base dimers, replication forks rarely disassemble in vivo, or visibly lose replicative polymerases.

Interestingly, cells contained multiple PolA-mV or ExoR-mV foci, both after treatment with MMC or after UV irradiation. In many cells, one or two foci (out of up to 10 foci in total) colocalized with DnaX-CFP, suggesting that both proteins might get recruited to forks during replication stress/DNA repair. We will come back to this point below. Clearly though, most PolA-mV or ExoR-mV foci did not colocalize with replication forks after damage induction, indicating that PolA and ExoR act at many chromosomal sites to remove DNA crosslinks and base modifications, independently from replication forks.

DnaE and PolC show very similar dynamics at the single molecule level.

We wished to further investigate if ExoR shows similar subcellular patterns of movement as PolA, b) if PolA plays its essential function at B. subtilis replication forks, c) if ExoR might complement a function of PolA at the forks, and d) how PolA behaves relative to PolC and DnaE. Epifluorescence fails to visualize very short events of molecules resting at a subcellular site (dwell time) and cannot detect mobile/diffusive molecules. We therefore employed single molecule tracking (SMT), which can follow the movement of single mVenus-fused proteins. We are using YFP-type SMT, in which most mVenus fusions are bleached until few molecules remain that can be tracked. We prefer this technique over PALM tracking, because blue light (as required for
PALM tracking of fluorophores PAmCherry or mEOS) arrests the *B. subtilis* cell cycle (unpublished results) and in our hands, *B. subtilis* cells show very high red auto-fluorescence under intensive excitation with 561 nm in cells lacking any FP fusion, which is not the case for 514 nm excitation required for mVenus excitation. DNA polymerase-mV fusions and ExoR-mV were tracked with 20 ms streams (50 fps), which yielded characteristic patterns of movement shown movies S1 and S2, and in suppl. Fig. S4: molecules could be static for several frames (Fig. S4a), which is defined as movement of less than 230 nm (Fig. S4d and e) or were mobile (Fig. S4f). The presence of at least two distinct fractions of stationary and mobile molecules can be seen in movies S1 and S2. No more than 3 signals per cell were allowed to ensure correct tracking, and single molecules were identified due to one step bleaching events (Fig. S4b and g). In order to obtain 2D patterns of movement, tracks obtained from many cells were projected into a standardized 3 x 1 µm large cell (Fig. S4d and i).

We used a Gaussian mixture model (GMM) to fit the probability density distribution function of all displacements from frame to frame. A single, freely diffusive population of molecules could be explained by a single Gaussian distribution (see, e.g. PfkA enzyme in (47)). However, for polymerases, a superposition of two Gaussians (red curves in Fig. 3 and Fig. 4) was necessary to fit to the experimental displacement distributions. The algorithm infers two diffusion constants, $D_1$ and $D_2$, corresponding to one fraction of immobile and another fraction of mobile molecules (Fig. 3). In case of DNA polymerases, $D_1$ would refer to the active, tightly DNA bound molecules, and $D_2$ to molecules being in the diffusive state. From fitting the variances and respective areas under the two Gaussians, we then determined the diffusion constants and relative fractions of molecules in the mobile and immobile states. The changes in the width of the step size distributions are a convenient visual tool to see if molecules become more static (the distribution becomes narrower) or more dynamic (wider distribution). The size of the bubbles indicates the fraction sizes, and the lower bubble corresponds to the static fraction having a lower diffusion constant (shown on the y-axis).
Interestingly, PolC and DnaE showed relatively similar dynamics: for PolC 52% of the molecules were in a slow/static mode with $D_{1,\text{static}} = 0.019 \pm 0.005 \mu m^2/s$ compared to 56% of DnaE molecules with $D_{1,\text{static}} = 0.015 \pm 0.004 \mu m^2/s$. 48% PolC molecules moved quickly, likely feely diffusive mode, with $D_{2,\text{mobile}} = 0.461 \pm 0.006 \mu m^2/s$, and 44% for DnaE with $D_{2,\text{mobile}} = 0.498 \pm 0.007 \mu m^2/s$. (Fig. 3, Table 1). This distribution cannot be explained with PolC being exclusively engaged in continuous leading strand synthesis, because only one out of the ten or more proteins per cell would be engaged in a static mode.

**PolA and ExoR dynamics change similarly after induction of DNA damage.**

Intriguingly, PolA and ExoR showed considerably different patterns of movement compared to PolC and DnaE: in the absence of DNA damage, PolA and ExoR consisted of a much smaller static fraction, and predominantly of mobile molecules, with PolA diffusing with a constant of $D_{1,\text{static}} = 0.022 \pm 0.007 \mu m^2/s$ (23%) and ExoR $D_{1,\text{static}} = 0.024 \pm 0.005 \mu m^2/s$ (28%) (Table 1, Fig. 4A and B). These experiments reveal that PolC and DnaE have similar static fractions, while PolA and ExoR show only small fractions that would correspond to a DNA-bound state under exponential growth conditions. This picture changed dramatically for PolA and for ExoR, when DNA damage was induced (Table 1). Here, the static fractions of both of PolA as well as ExoR molecules increased by about 50% (PolA: 75% static with $D_{1,\text{static}} = 0.013 \pm 0.003 \mu m^2/s$ and ExoR: 72% static with $D_{1,\text{static}} = 0.012 \pm 0.005 \mu m^2/s$) after MMC-treatment, and similarly, 70% PolA enzymes were static ($D_{1,\text{static}} = 0.015 \pm 0.003 \mu m^2/s$) or 72% of ExoR ($D_{1,\text{static}} = 0.016 \pm 0.006 \mu m^2/s$) following UV-treatment. In agreement with the epifluorescence data, PolC and DnaE showed minor changes in their mobility upon addition of MMC or treatment with UV: 55% of PolC molecules ($D_{1,\text{static}} = 0.024 \pm 0.008 \mu m^2/s$) and 49% of DnaE $D_{1,\text{static}} = 0.022 \pm 0.004 \mu m^2/s$ were static (MMC-treatment) and 49% of PolC ($D_{1,\text{static}} = 0.025 \pm 0.006 \mu m^2/s$) and 47% of DnaE ($D_{1,\text{static}} = 0.026 \pm 0.006 \mu m^2/s$) after UV-irradiation, showing that only few replicative DNA polymerase molecules are lost from the replication forks within the population.
We also used apparent diffusion analyses as a second tool to determine diffusion constants and used the probability density function (pdf) to analyse if populations with distinct diffusion constants exist (40). Fig. 5 illustrates well that static fractions (red) of PolC and of DnaE do not vary in response to DNA damage, and are similar to each other, while static fractions of PolA and of ExoR change dramatically, and in a very similar manner, after damage induction. These analyses reinforce the findings that PolC and DnaE do not strongly change their diffusive/bound distribution after addition of MMC or after UV treatment (Fig. 3), while the distribution of PolA and of ExoR changes in a strong and similar manner (Fig. 4).

PolA and ExoR influence each other’s dynamics after DNA damage induction in an opposing manner

In order to investigate if the PolA and ExoR change their dynamics when the other protein is missing, we performed SMT in a strain expressing PolA-mV and lacking ExoR, or vice versa. Interestingly, we observed significant differences between fraction sizes: in the absence of ExoR, PolA-mV was less static after addition of MMC or after UV irradiation (Fig. 4C: this can be easiest seen from the dashed line showing an increased dynamic fraction in the \(\text{exoR} \) mutant), while ExoR-mV became more static in the absence of PolA during DNA repair (Fig. 4D). In non-stressed cells, PolA or ExoR did not show considerable differences in their mobility in the absence of the other protein. These experiments show that PolA and ExoR have an effect on each other, implying some degree of interdependence of activity. It appears that PolA spends less time at damaged sites in the presence of ExoR, maybe because ExoR takes over after PolA activity, while ExoR appears to need PolA to spend an appropriate time at damage sites and is less engaged in repair when PolA is missing. However, alternative interpretations are possible.

PolC and DnaE show very similar dwell times at the forks.

Apparent diffusion and GMM analyses show that PolC and DnaE have similar static and dynamic fractions, but they do not allow conclusions about exchange rates of molecules at the replication forks. To analyse this, we firstly
investigated DnaX as a marker for the forks, with the rationale that DnaX dwell times at forks should be longer than those of DnaE, because the latter is expected to be exchanged with every new priming event, while this is likely not the case for DnaX. Fig. 6 shows that DnaX has a considerably higher static fraction (80% with $D_{1,\text{static}} = 0.010 \pm 0.004 \, \mu m^2/s$) than PolC and DnaE (Table 1). We next scored the number of molecules in PolC-mV, DnaE-mV and DnaX-mV expressing cells that are immobile for a certain number of consecutive acquisition times, deduced from the probability that a molecule will remain inside a circle of a certain radius dependent on time $t$.

Dwell times are estimated using an exponential decay model, and the best result we obtained for all proteins employed a 2 components model (see Fig. S5 for an example of PolA-mV), resulting in two distinct average dwell times, $\tau_1$ and $\tau_2$ (Table 2), indicative of molecules having short dwell times (every freely diffusing molecule will stop once in a while), and of molecules tightly binding somewhere in the cell: in this case to replication forks, which is the average dwell time of importance here. PolC and DnaE molecules dwelled on average $\tau_{2,\text{PolC}} = 1.40 \pm 0.12 \, s$, and $\tau_{2,\text{DnaE}} = 1.76 \pm 0.35 \, s$ (Table 2, Fig. 7), showing that they indeed come on and off the forks in a similar temporal manner. This can also be seen when their dwell times are compared with that of DnaX, $\tau_{2,\text{DnaX}} = 2.64 \pm 0.30 \, s$ (Table 2), because DnaX would be expected to stay longer at the forks compared to (a) lagging strand polymerase(s). Our dwell times are in good agreement with single molecule experiments for PolC from the Biteen group (48).

In agreement with some PolC and DnaE molecules leaving stalled forks in response to DNA damage, average dwell times became significantly shorter after addition of MMC, as indicated by a two-sample Kolmogorov-Smirnov test, for PolC from $1.40 \pm 0.12 \, s$ to $1.11 \pm 0.54 \, s$ (or $1.05 \pm 0.23 \, s$ after UV irradiation), and for DnaE from $1.76 \pm 0.35 \, s$ to $1.2 \pm 0.50 \, s$ (or to $1.02 \pm 0.48 \, s$ after UV treatment) (Fig. 7). Conversely, DnaX continued to remain at replication forks irrespective of the induction of DNA damage (Fig. 7, Table 2).

For PolA and ExoR, $\tau_2$ average dwell times increased markedly, from $1.20 \pm 0.20 \, s$ during exponential growth to $2.03 \pm 0.48 \, s/1.76 \pm 0.25 \, s$ after
MMC/UV treatment for PolA (Table 1), and in a very similar manner for ExoR (Fig. 7), revealing strongly increased DNA binding in response to DNA damage. These experiments not only verify that we can accurately detect changes in dwell times for DNA polymerases at the forks, but also underline our findings that PolA and ExoR become strongly DNA-bound, at many sites on the chromosome, during DNA repair. Average dwell times of PolA and ExoR increased in a significant manner when one of the enzymes was missing (Fig. 7, Table 2); for PolA only during repair of UV-induced damage (from 1.76±0.25 to 2.20±0.48 s), and for ExoR during UV damage (1.65±0.54 to 2.03±0.55 s) and even during exponential growth (1.11±0.45 to 1.32±0.58 s). These findings support the notion that the two proteins are partially redundant and confer (an) overlapping essential function(s), because their in vivo diffusion/binding patterns are altered in the absence of each other.

PolA and ExoR are recruited to replication forks upon addition of DNA damage

From Figure 1, it appeared that PolA and ExoR are located throughout the cells, and are not associated with the nucleoids, while PolC and DnaE formed nucleoid-associated foci. In order to clarify if not even single PolA molecules are associated with the replication forks for short intervals, we tracked PolA-mV and ExoR-mV relative to DnaX-CFP. To understand how the motion varies with position within the cell, we developed a tool that allows to visualize the motion of tracked molecules relative to a defined position in the cell, in this case the replication fork (or one of the two visible DnaX-CFP foci), and mapped the step sizes of PolA-mV and ExoR-mV as a function of distance from the replisome DnaX-CFP. We first acquired the location of DnaX-CFP using 445 nm stream acquisition, which reveals the localization of static CFP molecules, and then tracked mVenus fusion molecules in the same cells; representative examples of cells growing exponentially or having been treated with MMC or UV are shown in Fig. 8A. Less than 5% of PolA-mV molecules showed static localization close to/at DnaX foci, corresponding to 85% of replication forks that did not show PolA-mV tracks. However, more than 90% of forks showed static PolA-mV tracks after addition of MMC or after UV
treatment (Fig. 8B), revealing that PolA becomes efficiently recruited to stalled replication forks. Very similarly, few to no ExoR molecules arrested at DnaX-CFP foci during unperturbed growth, while they clearly halted at forks after DNA damage induction (Fig. 8B). The fact that most positions corresponding to a replisome show the presence of a considerable number of static PolA or ExoR molecules rules out that absence of PolA from forks during exponential growth is due to our inability to visualize all PolA molecules at a single molecule level, and supporting the findings from epifluorescence experiments that both proteins arrest replication forks as well as at many chromosomal sites away from the replisome, during repair events. The fact that extremely few PolA or ExoR molecules arrest close to forks can be seen in Fig. 8B, upper panels, while the recruitment to one, two or three visible forks can be clearly seen in damaged cells (Fig. 8B, middle and lower panels), besides the effect that both PolA and ExoR become more statically positioned and less mobile after induction of DNA damage, in agreement with GMM and apparent diffusion analyses. Therefore, PolA is not recruited to replication forks during exponential growth in a similar manner as PolC or DnaE, questioning whether it plays a significant role during unperturbed replication. If PolA was to remove RNA/DNA hybrids at the lagging strand, we would have expected to see a considerable fraction of PolA molecules at the forks. Similarly, ExoR is also transiently recruited to replication forks during active DNA repair, but rarely present during exponential growth.

Heat maps can help to detect significant changes in binding patterns between different cellular states. Therefore, all localization events from hundreds of cells were plotted into a standardized 3 x 1 µm large cell. Fig. S6 shows that while PolC and DnaE mostly reside on the central part of the cells that is occupied by the nucleoid, PolA and ExoR are observed more diffusely throughout the cell without clear accumulations, except when DNA damage is induced; here, many more foci reside at places within the central part (i.e. the nucleoids) of the cells.
ExoR is a magnesium-dependent exonuclease and shows highest binding affinity to 5’ overhangs in dsDNA

ExoR is a small conserved protein of previously unknown function, and its sequence similarity is restricted to the N-terminal domain of PolA, which contains a 3’→5’ exonuclease motif shown to be functional in S. pneumoniae and E. coli (25,49,50). We performed structure modelling (51) based on the homology of ExoR to known exopolymerases, and found that ExoR can be superimposed with the exonuclease domain of Pol I (Fig. S7), revealing a close structural relation. For biochemical characterization, a 6His-ExoR construct was purified to homogeneity by Nickel-Sepharose affinity chromatography followed by size-exclusion chromatography (see Materials and Methods and Fig. S8). ExoR showed magnesium-dependent nuclease activity on double stranded DNA (Fig. 9), in agreement with its proposed structure. We wished to gain insight into preferred substrates of ExoR, and therefore performed DNA binding assays, in the absence of magnesium. Linear DNA fragments carrying regions of 68 bp or less (dsDNA, ssDNA and RNA) were mixed, and increasing amounts of ExoR of 0 to 750 nM molar excess with respect to DNA were added to the different substrates. As shown in the EMSA assays in Fig. 10, well-defined complexes were detected even at the lowest amount of ExoR used. Defined and single shifted bands were observed on all dsDNA, ssDNA and RNA molecules, indicating a distinct number of ExoR molecules interacting with the substrate, similarly to all polymerases containing the C-terminal exonuclease domain (52). ExoR had a clear preference for dsDNA with a 5’ overhang (Fig. 10A) over 3’ overhangs (Fig. 10E), over blunt end dsDNA (Fig. 10D). Interestingly, it bound more readily to an ssDNA template (Fig. 10B) than to the corresponding dsDNA, and also to ssRNA (Fig. 10C), while affinity to a DNA/RNA hybrid with a 5’ overhang was higher than to ssRNA or dsDNA (Fig. 10F). These results clearly show binding of ExoR to dsDNA is influenced by the DNA structure and support the idea that ExoR could extend single stranded gaps in DNA. Previous functional genomics studies have suggested that DNA polymerase I (PolA) is essential in S. pneumoniae (50), E. coli (49), and S. enterica (53) but not in B. subtilis (54), S. aureus (55), or H. influenzae (56), but that a double
exoR polA deletion is lethal. Our data suggest that exonuclease activity of either PolA or of ExoR must be present in *B. subtilis* to overcome damage-induced replication stress.
DISCUSSION

Many critical cell cycle processes are achieved by multienzyme complexes, in which the timely interplay of components is crucial for the correct functioning of the machinery. This is especially true for replication forks, where the synthesis of two DNA strands occurs in parallel, however in a continuous and discontinuous manner for leading and lagging strand. The molecular mechanism of DNA synthesis has been intensively investigated in vitro (57,58), revealing distinct mechanisms of leading and lagging strand synthesis. The advent of single molecule tracking (SMT) has made it possible to visualize the dynamics of single enzymes in real time, i.e. follow diffusion and binding/unbinding events in millisecond intervals, and with high spatial precision (down to 20 nm resolution). We have applied SMT to study Bacillus subtilis replication forks to study the dynamics of two replicative polymerases, a subunit of the clamp loader complex (DnaX), polymerase I (PolA) and a putative exonuclease, ExoR (YpcP).

Our experiments document that PolC and DnaE are recruited to and released from the replisome in a time scale of few seconds, with both polymerases exchanging with similar kinetics, but slower than DnaX, a part of the clamp loader complex (Fig. 11). If PolC was exclusively involved in leading strand synthesis, we would have expected much longer dwell times than those of DnaE, and a much lower fraction of proteins being involved in replication. Thus, our findings are in agreement with genetic and biochemical experiments showing that PolC and DnaE work together at the lagging strand. Because only DnaE can extend RNA primers but is a relatively slow and error-prone enzyme in vitro (1,14,59), PolC must take over DNA extensions made by DnaE. It had been speculated that DnaE only briefly carries out primer extension, and leaves elongation to PolC, which has proofreading activity. Our findings that PolC and DnaE exchange in a similar manner support the idea that DnaE is involved in both, RNA primer extension as well as in elongation, which becomes less error-prone in the presence of PolC, which directly interacts with DnaE (60). Our data show that PolC and DnaE operate with balanced dynamics at replication forks, supporting the findings of coordinated activity at the lagging strand (bearing in mind that leading strand
activity and exchange of PolC will hardly affect the measured \textit{in vivo} exchange rates).

A major activity of PolC during leading strand synthesis has been used to explain how the slow polymerase activity of DnaE (which is increased in the presence of DnaN and of PolC (60)) can be compensated for to keep up with the fast speed of leading strand synthesis. Interestingly, \textit{in vitro} single molecule experiments have shown that leading strand polymerase activity comprises stochastic stopping and slowing down events, and that DNA helicase can adapt its activity to slowed-down synthesis at the lagging strand (61). Thus, a model that stochastic hand-over to PolC of extended RNA/DNA strands generated by primase and then DnaE might work hand in hand with leading strand synthesis (Fig. 11) by adapting helicase speed and slowing down Pol III activity.

A second important finding is a surprising stability of replication forks in response to the induction of chemical damage to DNA, which is thought to lead to stalling of replication forks. Moreover, we found that only about 20% of PolC and DnaE are displaced from the forks during repair, possibly replaced by translesion polymerases. Our results show that the replication machinery is relatively robust against chemical insults, and that only a minor fraction of forks will exchange PolC and/or DnaE for translesion DNA polymerases, which can polymerize over non-canonical base pairs that are induced by e.g. UV irradiation (62). Interestingly, DnaE can also operate in an error-prone mode (17), and may even switch template to the lagging strand to bypass lesions (63). We would like to point out that in case the fluorescent protein fusions generated for this study were not fully functional, we would have expected high loss of PolC and DnaE from the forks, but the opposite was true, strongly suggesting that our observations closely reflect actual processes \textit{in vivo}.

A third major conceptual insight from our study is the scarce presence of polymerase I (PolA) at replication forks during normal replication (Fig. 11). If PolA were to remove RNA hybrids left in Okazaki fragments like Pol I in \textit{E. coli} cells, we would have expected a behaviour of PolA at least partially similar to that of DnaE. However, we found that few PolA molecules dwell at the
replication machinery during exponential growth, and a vast majority is either
statically positioned at various sites on the chromosome of diffusing through
the cell. Conversely, PolA become significantly enriched at forks after
induction of DNA damage, be it caused by chemical or physical (UV) insult.
These data strongly suggest that an enzyme other than PolA removes RNA
primers at the lagging strand, and that PolA performs an inducible repair
function at stalled forks. It is tempting to speculate that PolC might remove
RNA primers, based on studies on primer removal during Okazaki fragment
maturation in mammalian cells (64), an activity that can not be carried out by
DnaE. This process involves displacement of the primer by the replicating
DNA polymerase (Polδ) (65) when it reaches the 5’-end of the RNA primer of
the previously formed Okazaki fragment. Even in the absence of a helicase,
Polδ can synthesize a few nucleotides into the duplex region by strand
displacement synthesis, creating a short 5’-primer flap. This flap can then be
cleaved away by nucleases, creating a 5’-DNA end through which the Okazaki
fragments can be ligated. This could work in an analogous manner in B.
subtilis, which will be interesting to study.

A fourth important contribution of our study is the finding that similar to
PolA, ExoR showed a considerably different pattern of movement and binding
events in response to DNA damage. While during exponential growth, ExoR
was mostly diffusive or occasionally arrested at random places on the
nucleoids, engaged in repair processes that occur due to spontaneous
lesions, the protein was recruited to replication forks after damage induction,
concomitant with a strong increase in average dwell time and in the fraction of
immobile molecules. Heat maps of PolA and of ExoR showed a higher
concentration on the nucleoids during unperturbed growth, suggesting that
many molecules are engaged in a DNA-scanning mode for DNA lesions. After
UV irradiation or induction of interstrand crosslinks, both proteins showed an
increase in statically positioned molecules on the nucleoids, including
replication forks (Fig. 11). Our data suggest that ExoR and PolA perform a
redundant essential function at the replication forks, which becomes more
heavily required when DNA damage has occurred. To better characterize the
previously poorly characterized protein ExoR (YpcP), which likely has a
similar structure as the exonuclease domain of Pol I/PoIA, we purified ExoR to shows that it indeed has DNA nuclease activity, dependent on the presence of magnesium, and has highest affinity to dsDNA with 5' overhangs but can also binds to DNA/RNA hybrids. These experiments indicate that ExoR could work at DNA nicks or remove excess ssDNA during homologous recombination. Because ExoR and PolA differ significantly in their bound/mobile fractions or in their average dwell times in the absence of each other, the proteins appear to act in a partially redundantly redundant but interconnected manner. Based on the conservation of ExoR between Gram-positive and Gram-negative bacteria, ExoR could play an important role in DNA repair in a large variety of bacteria.

DnaE has been suggested to be involved in DNA repair (59) in addition to its vital contribution to the elongation phase of the lagging strand (14). However, if DnaE had a substantial role in DNA repair following damage with MMC or UV, we would have expected to observe increased dwells for DnaE. Because DnaE becomes less static in response to DNA damage, our findings suggest that the contribution of DnaE to DNA repair synthesis is only minor, compared to that of PolA.

In toto, we provide in vivo evidence that B. subtilis replication forks present interesting features of dynamics of replication proteins, which suggest that forks operate differently from the E. coli or eukaryotic systems, with a possible scenario being depicted in Fig. 11. It will be interesting to study the molecular basis of the division of labour at the lagging strand between the two replicative polymerases, and the exact function of ExoR at replication forks.

Acknowledgments

This work was supported by a grant 229388 from The National Council of Science and Technology of Mexico, in the postdoctoral stays abroad program, and by the Center for Synthetic Microbiology at the Philipps-Universität Marburg, funded by the LOEWE Program of the state of Hessen.
Author contributions
R. H.-T. performed all experiments, evaluated data and co-wrote the manuscript, L.M.O.B. devised of SMT methods and helped analyse the data, G.F. conceived of the GMM methods, helped in data analyses and co-wrote the manuscript, P.L.G. conceived of the study and co-wrote the manuscript.

Competing interests
The authors declare no financial of scientific competing interests.
REFERENCES


<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of cells</th>
<th>No. of tracks</th>
<th>$D$ ($\mu m^2 \cdot s^{-1}$)</th>
<th>$D_1$ ($\mu m^2 \cdot s^{-1}$)</th>
<th>$F_1$ (%)</th>
<th>$D_2$ ($\mu m^2 \cdot s^{-1}$)</th>
<th>$F_2$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Damage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PolC-mV</td>
<td>123</td>
<td>3572</td>
<td>0.226 ± 0.004</td>
<td>0.019 ± 0.005</td>
<td>52</td>
<td>0.461 ± 0.006</td>
<td>48</td>
</tr>
<tr>
<td>DnaE-mV</td>
<td>112</td>
<td>3976</td>
<td>0.242 ± 0.005</td>
<td>0.015 ± 0.004</td>
<td>56</td>
<td>0.498 ± 0.007</td>
<td>44</td>
</tr>
<tr>
<td>PolA-mV</td>
<td>156</td>
<td>4213</td>
<td>0.398 ± 0.007</td>
<td>0.022 ± 0.007</td>
<td>23</td>
<td>0.600 ± 0.003</td>
<td>77</td>
</tr>
<tr>
<td>ExoR-mV</td>
<td>138</td>
<td>3765</td>
<td>0.357 ± 0.003</td>
<td>0.024 ± 0.005</td>
<td>28</td>
<td>0.656 ± 0.008</td>
<td>72</td>
</tr>
<tr>
<td>DnaX-mV</td>
<td>126</td>
<td>4023</td>
<td>0.123 ± 0.005</td>
<td>0.010 ± 0.004</td>
<td>80</td>
<td>0.420 ± 0.002</td>
<td>20</td>
</tr>
<tr>
<td>$\Delta$exoR PolA-mV</td>
<td>132</td>
<td>3567</td>
<td>0.334 ± 0.005</td>
<td>0.023 ± 0.002</td>
<td>24</td>
<td>0.512 ± 0.006</td>
<td>76</td>
</tr>
<tr>
<td>$\Delta$polA ExoR-mV</td>
<td>142</td>
<td>3952</td>
<td>0.358 ± 0.008</td>
<td>0.020 ± 0.001</td>
<td>23</td>
<td>0.589 ± 0.005</td>
<td>77</td>
</tr>
<tr>
<td>MMC-treated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PolC-mV</td>
<td>135</td>
<td>4623</td>
<td>0.246 ± 0.005</td>
<td>0.024 ± 0.008</td>
<td>55</td>
<td>0.512 ± 0.004</td>
<td>45</td>
</tr>
<tr>
<td>DnaE-mV</td>
<td>123</td>
<td>3987</td>
<td>0.267 ± 0.005</td>
<td>0.022 ± 0.004</td>
<td>49</td>
<td>0.503 ± 0.005</td>
<td>51</td>
</tr>
<tr>
<td>PolA-mV</td>
<td>145</td>
<td>3890</td>
<td>0.313 ± 0.007</td>
<td>0.013 ± 0.003</td>
<td>75</td>
<td>0.610 ± 0.008</td>
<td>25</td>
</tr>
<tr>
<td>ExoR-mV</td>
<td>134</td>
<td>4213</td>
<td>0.297 ± 0.004</td>
<td>0.012 ± 0.005</td>
<td>72</td>
<td>0.580 ± 0.004</td>
<td>28</td>
</tr>
<tr>
<td>DnaX-mV</td>
<td>149</td>
<td>3657</td>
<td>0.259 ± 0.004</td>
<td>0.010 ± 0.006</td>
<td>76</td>
<td>0.651 ± 0.002</td>
<td>24</td>
</tr>
<tr>
<td>$\Delta$exoR PolA-mV</td>
<td>124</td>
<td>3418</td>
<td>0.222 ± 0.007</td>
<td>0.014 ± 0.008</td>
<td>73</td>
<td>0.598 ± 0.003</td>
<td>27</td>
</tr>
<tr>
<td>$\Delta$polA ExoR-mV</td>
<td>129</td>
<td>4127</td>
<td>0.239 ± 0.006</td>
<td>0.011 ± 0.004</td>
<td>75</td>
<td>0.618 ± 0.005</td>
<td>25</td>
</tr>
<tr>
<td>UV-treated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PolC-mV</td>
<td>131</td>
<td>4098</td>
<td>0.224 ± 0.007</td>
<td>0.025 ± 0.005</td>
<td>49</td>
<td>0.499 ± 0.003</td>
<td>51</td>
</tr>
<tr>
<td>DnaE-mV</td>
<td>127</td>
<td>4100</td>
<td>0.218 ± 0.005</td>
<td>0.026 ± 0.006</td>
<td>47</td>
<td>0.512 ± 0.004</td>
<td>53</td>
</tr>
<tr>
<td>PolA-mV</td>
<td>133</td>
<td>3765</td>
<td>0.236 ± 0.007</td>
<td>0.015 ± 0.003</td>
<td>70</td>
<td>0.630 ± 0.009</td>
<td>30</td>
</tr>
<tr>
<td>ExoR-mV</td>
<td>128</td>
<td>4021</td>
<td>0.213 ± 0.004</td>
<td>0.016 ± 0.006</td>
<td>72</td>
<td>0.587 ± 0.007</td>
<td>28</td>
</tr>
<tr>
<td>DnaX-mV</td>
<td>132</td>
<td>4402</td>
<td>0.131 ± 0.007</td>
<td>0.011 ± 0.006</td>
<td>68</td>
<td>0.612 ± 0.003</td>
<td>32</td>
</tr>
<tr>
<td>$\Delta$exoR PolA-mV</td>
<td>147</td>
<td>3210</td>
<td>0.211 ± 0.003</td>
<td>0.013 ± 0.004</td>
<td>72</td>
<td>0.543 ± 0.005</td>
<td>28</td>
</tr>
<tr>
<td>$\Delta$polA ExoR-mV</td>
<td>134</td>
<td>3672</td>
<td>0.201 ± 0.005</td>
<td>0.012 ± 0.008</td>
<td>76</td>
<td>0.564 ± 0.007</td>
<td>24</td>
</tr>
</tbody>
</table>

a $D$, average diffusion constant of all molecules.

b $D_1$, diffusion constant of static fraction.

c $F_1$, percentage of static molecules.

d $D_2$, diffusion constant of mobile fraction.

e $F_2$, percentage of mobile molecules.
<table>
<thead>
<tr>
<th>No Damage</th>
<th>UV</th>
<th>MMC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PolC-mV</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\tau_1$</td>
<td>0.058±0.05</td>
<td>0.053±0.07</td>
</tr>
<tr>
<td>$\tau_2$</td>
<td>1.40±0.12</td>
<td>1.051±0.23</td>
</tr>
<tr>
<td><strong>DnaE-mV</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\tau_1$</td>
<td>0.054±0.05</td>
<td>0.043±0.09</td>
</tr>
<tr>
<td>$\tau_2$</td>
<td>1.76±0.35</td>
<td>1.02±0.48</td>
</tr>
<tr>
<td><strong>DnaX-mV</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\tau_1$</td>
<td>0.063±0.03</td>
<td>0.042±0.05</td>
</tr>
<tr>
<td>$\tau_2$</td>
<td>2.64±0.30</td>
<td>2.40±0.35</td>
</tr>
<tr>
<td><strong>PolA-mV</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\tau_1$</td>
<td>0.045±0.03</td>
<td>0.045±0.15</td>
</tr>
<tr>
<td>$\tau_2$</td>
<td>1.20±0.20</td>
<td>1.76±0.25</td>
</tr>
<tr>
<td><strong>ExoR-mV</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\tau_1$</td>
<td>0.040±0.04</td>
<td>0.045±0.03</td>
</tr>
<tr>
<td>$\tau_2$</td>
<td>1.11±0.45</td>
<td>1.65±0.54</td>
</tr>
<tr>
<td><strong>ExoR-mV $\Delta polA$</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\tau_1$</td>
<td>0.045±0.04</td>
<td>0.046±0.03</td>
</tr>
<tr>
<td>$\tau_2$</td>
<td>1.32±0.58</td>
<td>2.03±0.55</td>
</tr>
<tr>
<td><strong>PolA-mV $\Delta exoR$</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\tau_1$</td>
<td>0.052±0.04</td>
<td>0.048±0.04</td>
</tr>
<tr>
<td>$\tau_2$</td>
<td>1.15±0.52</td>
<td>2.20±0.48</td>
</tr>
</tbody>
</table>


**LEGENDS TO THE FIGURES**

**Fig. 1** Localization of polymerases during exponential growth. Subcellular localization by epifluorescence in representative untreated live *B. subtilis* cells. The overlay panels show DnaX-CFP in red and PolC-mV, DnaE-mV, PolA-mV and ExoR-mV in green. Scale bar: 2 µm.

**Fig. 2** Altered localization patterns after induction of DNA damage. Localization by epifluorescence in representative live *B. subtilis* cells after induction of DNA damage with MMC. The overlay panels show DnaX-CFP in red and PolC-mV, DnaE-mV, PolA-mV, and ExoR-mV in green. Note that DnaX-CFP foci rarely colocalize with PolA-mV or ExoR-mV foci. Scale bar: 2 µm.

**Fig. 3** Diffusion patterns of DNA polymerases. Gaussian mixture model (GMM) analyses of frame-to-frame displacements in x- and y-directions. A) PolC-mV B) DnaE-mV. Red lines represent the sum of the two Gaussian distributions. Dotted and dashed lines represent the single Gaussian distributions corresponding to the static and mobile fractions. Bubble plots show a comparison of fraction sizes (size of the bubble) and diffusion constants (y-axis), between different growth conditions: distribution in untreated cells (dark blue circles), in MMC-treated (yellow) and in UV-treated (light blue circles) cells. Step size distributions reveal two populations for each protein, a mobile (upper circles) and a static (lower circles) fraction.

**Fig. 4** Diffusion patterns of PolA and of ExoR. GMM analyses of frame-to-frame displacements in x- and y-directions. A) PolA-mV, B) ExoR-mV, C) PolA-mV ΔexoR D) ExoR-mV ΔpolA. See Fig. 3 for explanations.

**Fig. 5** Apparent diffusion of polymerases. Histograms of apparent diffusion coefficients (D<sub>i</sub>) of PolC-mV, DnaE-mV, PolA-mV and ExoR-mV before and after treatment with MMC and UV. Distributions show events of resting and
movement fitted by a two-component exponential function. Red lines indicate the static fraction and blue lines the dynamic population.

Fig. 6 Apparent diffusion and bubble plots of DnaX-mV compared to PolC-mV and DnaE-mV. Distribution of apparent diffusion coefficients for DnaX-mV trajectories. Histograms show events of resting and movement fitted by a two-component exponential function. Red lines mark the static fractions and blue lines dynamic populations. Bubble plots illustrate fraction sizes and corresponding diffusion coefficients for DnaX-mV, PolC-mV and DnaE-mV in untreated cells.

Fig. 7 Dwell times. Cumulative distribution of residence times of PolC-mV, DnaE-mV, PolA-mV, ExoR-mV, ExoR-mV ΔpolA and PolA-mV ΔexoR strains, before and after treatment with MMC or UV. Histograms show events of resting fitted by a two-component exponential function. Error bars derive from bootstrapping. Bars pointing left represent short dwell times included in random motion, bars to the right long dwell times of DNA-bound molecules. Dark blue bars untreated cells, yellow MMC-treated cells, light blue UV-treated cells.

Fig. 8 Localization of PolA and ExoR relative to replication forks. A) Location of DnaX-CFP and overlaid tracks from PolA-mV or from ExoR-mV in representative B. subtilis cells untreated and after induction of DNA damage with MMC or UV light as indicated. B) Distance of PolA-mV or ExoR-mV tracks relative to a DnaX-CFP focus, which is positioned at “0 µm”. Panels correspond to those in (A), such that the position of a second or third DnaX-CFP focus can be inferred after induction of DNA damage by the additional accumulation of static tracks far from the “0” position.

Fig. 9 Exonuclease activity of ExoR depends on the presence of magnesium. PstI-cut fluorescently labelled pUC19 DNA (2.3 nM) was incubated with increasing amounts of purified ExoR for 30 min. The reaction was quantified by scanning of the emission between 510 and 550 nm (see
Supplementary Methods). (A) In the presence of magnesium, curves 1 through 8 contained 0, 20, 40, 60, 80, 120, 180, and 240 nM ExoR, respectively. The dsDNA curve presents 0 mM of ExoR, the ssDNA curve presents data from a reaction mixture that contained 0.5 equivalents of denatured input DNA to imitate limited digest conditions. (B) as in A) but in the absence, of magnesium.

**Fig. 10 DNA binding of ExoR.** EMSAs showing ExoR binding to different nucleotide substrates. ExoR binding specifically to dsDNA panels A, D and E or ssDNA (panel B), or to RNA (panel C and F). EMSAs were performed with increasing amounts (0–750 nM) of purified ExoR and fragments of 68 bp, containing either DNA or RNA, generated by annealing of custom-made oligonucleotides. Samples were mixed with loading buffer and analysed through 6% (v/v) native polyacrylamide gels. Lanes labelled “0” show the control substrate in the absence of protein. ExoR shows highest binding affinity to 5’ overhangs in dsDNA (panel A). Lines below each panel represent DNA in blue or RNA in red.

**Fig. 11 Model for dynamics of proteins at Bacillus subtilis replication forks.** During exponential growth (left cartoon), PolC (DNA Pol III) polymerises the leading strand (on the right), while primase synthesizes short RNA primers at the lagging strand (left), which are first extended by DnaE and in a putative hand-over mechanism by PolC. Polymerases at the lagging strand are newly loaded each few thousand base pairs by the clamp loader complex, while helicase unwinds the DNA duplex ahead of the polymerases. Note that single strand binding protein SSB is not shown for simplicity reasons. Newly synthesized DNA strands are indicated by dashed lines. When replications fork run into damaged DNA (indicated by the flash), ExoR and/or PolA (DNA polymerase I) arrest at forks and play an important role in removing damaged sites and in restarting replication forks.
Fig. 1
Fig. 2

A  PolC-mV

B  DnaE-mV

Fig. 3
Fig. 4
Fig. 5
Fig. 6
Fig. 7
**A**

PolA-mV DnaX-CFP  |  ExoR-mV DnaX-CFP

- **Overlay Tracks**
  - No Damage
  - MMC 50 ng/ml
  - UV 45 J/cm²

**B**

- **PolA-mV DnaX-CFP**
  - No Damage
  - MMC 50 ng/ml
  - UV 45 J/cm²

- **ExoR-mV DnaX-CFP**
  - No Damage
  - MMC 50 ng/ml
  - UV 45 J/cm²

---

1050 1051  

**Fig. 8**
Fig. 9
Fig. 10
Fig. 11