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Recombinase and translesion DNA polymerase decrease the speed of replication fork progression during the DNA damage response in *Escherichia coli* cells

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ABSTRACT

The SOS response is a DNA damage response pathway that serves as a general safeguard of genome integrity in bacteria. Extensive studies of the SOS response in *Escherichia coli* have contributed to establishing the key concepts of cellular responses to DNA damage. However, how the SOS response impacts on the dynamics of DNA replication fork movement remains unknown. We found that inducing the SOS response decreases the mean speed of individual replication forks by 30–50% in *E. coli* cells, leading to a 20–30% reduction in overall DNA synthesis. *dinB* and *recA* belong to a group of genes that are upregulated during the SOS response, and encode the highly conserved proteins DinB (also known as DNA polymerase IV) and RecA, which, respectively, specializes in translesion DNA synthesis and functions as the central recombination protein. Both genes were independently responsible for the SOS-dependent slowdown of replication fork progression. Furthermore, fork speed was reduced when each gene was ectopically expressed in SOS-uninduced cells to the levels at which they are expressed in SOS-induced cells. These results clearly indicate that the increased expression of *dinB* and *recA* performs a novel role in restraining the progression of an unperturbed replication fork during the SOS response.

INTRODUCTION

Cells are constantly exposed to DNA damage produced by various endogenous and exogenous agents such as reactive oxygen species, UV radiation and ionizing radiation. Failure of the cell to properly respond to DNA damage can lead to genetic disorder. To sustain cell viability and protect genome integrity when their DNA is damaged, all organisms have evolved DNA damage response systems, the best characterized being DNA damage checkpoints in eukaryotes and the SOS response in bacteria (1). When DNA replication is perturbed in eukaryotes, the intra-S-phase checkpoint delays the progression of DNA replication by globally inhibiting the firing of replication origins that have not yet been initiated (2). There is some evidence that the intra-S-phase checkpoint also actively slows the progression of normal ongoing replication forks (3–7). Progress has been made in elucidating how the checkpoint shuts down origin firing (8), although the direct effector molecules and the molecular mechanisms underlying replication fork slowing have not been precisely clarified due to the complexity of checkpoint signalling pathways.

The SOS response of *Escherichia coli* is a simple and well-characterized DNA damage response. The bacterial system is controlled by the regulator genes *recA* and *lexA*, which encode the main recombinase and SOS-repressor proteins, respectively (9). Single-stranded DNA (ssDNA) regions accumulate at replication forks arrested by DNA damage (10,11) and become coated with single-stranded DNA binding protein (SSB), onto which RecA is loaded by recombination mediator proteins to form a nucleoprotein filament that activates the co-protease function of RecA (12). This activated form of RecA (RecA*) facilitates autocleavage of LexA bound to the operator region, leading to the derepression of more than 40 SOS genes including *recA*, *umuDC, dinB* and *sulA* (13), and consequently to the activation of DNA repair, damage tolerance and cell cycle delay.

The term ‘checkpoint’, derived from research in eukaryotes, has also been applied to DNA replication during the bacterial SOS response (14). The *umuDC*-dependent dam-

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age checkpoint delays the resumption of DNA synthesis after DNA damage to allow the cell more time to repair DNA damage (15). Based on the following findings, we and others have proposed DinB (also called DNA polymerase (Pol) IV) as a candidate effector that delays DNA replication in a bacterial counterpart of the eukaryotic intra-S-phase checkpoint. Excess DinB directly inhibited a moving replicative Pol III that was rapidly catalysing chain elongation on the template DNA, by detaching Pol III* (Pol III lacking the β clamp subunit) from the β clamp and taking over DNA synthesis from Pol III in a concentration-dependent manner *in vitro* (16–19). Consistent with these biochemical studies, ectopic overexpression of *dinB* inhibited DNA replication in a dose-dependent manner *in vivo* (18,20,21). Furthermore, substantial overproduction of DinB abolished replication fork progression at random genomic locations (20,21). However, the fork-brake activity of DinB has not been evaluated in cells expressing the SOS response, and because of difficulties in accurately measuring replication fork speed in *E. coli* cells, it remains unknown whether the SOS response itself affects the dynamics of moving replication forks.

We have recently constructed a new thymidine-requiring strain named eCOMB (*E. coli* for combing), which enables the accurate measurement of replication fork speed in DNA combing experiments with *E. coli* cells (22). In fork speed determination with eCOMB cells undergoing the DNA damage response, it is important to be able to distinguish between the passive effect of DNA lesions that act as obstacles to the replication machinery and an active effect of the SOS response on slowing replication fork progression (23). To accomplish this, a 'physiological' SOS response that is independent of exogenous DNA damage was induced in eCOMB cells carrying mutations in the regulator genes of the SOS response. Here, we report that *E. coli* cells significantly reduce the speed of unperturbed replication forks during the physiological SOS response. We also identified not only *dinB* but also *recA* as being responsible for the reduced speed of replication forks in the bacterial damage response, which represents a new role for both genes in the management of replication fork speed in the SOS response. This function appears to represent the bacterial equivalent of an active control in the dynamics of DNA replication fork behaviour that has been observed in the eukaryotic intra-S-phase checkpoint. Our findings suggest that slowdown of replication fork movement is a general feature of the damage response during DNA replication.

MATERIALS AND METHODS

Chemicals and antibodies

The thymidine analogue 5-iodo-2′-deoxyuridine (IdU) was purchased from Sigma-Aldrich, USA, and the analogue 5-chloro-2′-deoxyuridine (CldU) from MP Biomedicals, USA. *o*-nitrophenol-*β*-D-galactopyranoside (ONPG) was purchased from Wako Pure Chemical Industries, Japan. [2–^{14}C] thymidine (>50 mCi mmol^{-1}) and [methyl-^{3}H] thymidine (70–90 Ci mmol^{-1}) were purchased from PerkinElmer, USA. Rabbit anti-RecA antibodies were purchased from Bio Academia, Japan. Mouse anti-BrdU monoclonal antibody B44 and rat anti-BrdU monoclonal antibody BU1/75 (ICR1) were purchased from Becton Dickinson, USA and Abcam, USA, respectively. Rabbit anti-DinB antibody was obtained from Dr Takehiko Nohmi (National Institute of Health Sciences, Japan) (24).

Media

We routinely supplemented 56/2 minimal medium (25) with 0.2% casamino acids and 20 μg ml^{-1} tryptophan as described previously (22). The 56/2 medium contained 2 μg ml^{-1} thymidine to grow eCOMB and its derivatives. To pulse-label cells for fork speed determination, thymidine in the medium was replaced by 50 μg ml^{-1} of halogenated thymidine analogue, either CldU or IdU as described in each experiment. LB and M9 salts were prepared as described elsewhere (26).

Construction of bacterial strains

All strains used in this study are derivatives of *E. coli* K12 (Supplementary Table S1). Plasmids and oligonucleotides used for strain construction are listed in Supplementary Tables S2 and S3, respectively. Replacement of a chromosomal gene by the kanamycin resistance (*kan*) gene was performed by P1(*vir*)-mediated transduction (27) with the respective donor from the Keio Collection, except for *umuDC* and *lexA* (28). A DNA fragment carrying Δ*umuDC*::FRT-*kan* was amplified by PCR using the pKD13 plasmid as template and the oligonucleotides dKm-F2 and dKm-R2. The *umuDC* gene of the BW25113 strain carrying the pKD46 plasmid was replaced with the polymerase chain reaction (PCR) product, using the Red-mediated targeting method (29), yielding MK7004. Likewise, a DNA fragment carrying Δ*lexA*::FRT-*kan* was amplified by PCR using pKD13 and the oligonucleotides JW4003-KN and JW4003-KC. The *lexA* gene of the MK7453 (eCOMB Δ*sulA*) strain carrying pKD46 was also replaced with the PCR product, resulting in MK7456. The MK7004 and MK7456 strains were used as donor strains to replace *umuDC* and *lexA*, respectively, by P1 transduction. Following each replacement of the chromosomal genes by *kan*, with the exception of *lexA*, the resulting strains were transformed with plasmid pCP20 to eliminate the *kan* gene at the flippase recognition target (FRT) by the site-specific flippase recombinase (29). No undesired cross-recombination at the remaining FRT scars was detected in any strain after the excision of *kan*. To construct MK7486, MK7496 and MK7498, the corresponding recipient Δ*recA* cells harbouring plasmid pRECA1 (Supplementary Table S2) were used in P1 transduction experiments. After P1 transduction, pRECA1 was eliminated by incubating the cells at 42°C. The *sulA*::*lacZ′YA* mutation of SY2 (30) was cotransduced with the closely linked *kan* into the eCOMB (22), SMR7467 and SMR7623 (31) strains, yielding MK7922, MK7925 and MK7926, respectively. The temperature-sensitive *recA441* of RM112 (32) was transduced with the tetracycline resistance gene of *srl300*::Tn*10* into the recipient MK7922 strain to create MK7933. The *lexA3* (Ind^{-}) allele encoding a non-cleavable LexA protein of SMR7467 (31) was transduced into the MK7933 strain by selecting for chloramphenicol resistance from the linked *malB*::Tn*9*, and MK7961 was obtained.

Construction of plasmids

General methods for DNA manipulation and transformation followed standard procedures (26). Entire inserts in all plasmids created in this study (Supplementary Table S2) were sequenced to verify that no mutations had been introduced during PCR amplification. Oligonucleotides used for PCR are listed in Supplementary Table S3. To construct plasmid pSCG3, carrying operator-constitutive *dinBo-21* (33), two parts of the *dinB* sequence were amplified from genomic DNA of MG1655 by PCR with the two primer pairs dinB-BF2X and dinB-MR2, and dinB-BR3X and dinB-MF2; dinB-MF2 and dinB-MR2 have a partially complementary sequence containing a mutation. The hybrid DNA of the two PCR products was amplified by recombinant PCR (34) with primers dinB-BF2X and dinB-BR3X. For subsequent cloning, the resulting DNA fragment of *dinBo-21* was digested with *Eco*RI and *Bam*HI, and ligated to the *Eco*RI and *Bam*HI sites of the single-copy vector pCC1BAC (Epicentre, USA) to obtain pSCG3. Likewise, the *recA* gene was amplified from MG1655 genomic DNA by PCR with two mutagenic primers, recA-MR1 and recA-MF1, in conjunction with primers recA-BF1 and recA-BR1 (Supplementary Table S3). Plasmid pSCG11, carrying the operator-constitutive *recAo* allele, was constructed by combining the two PCR products and linearized pCC1BAC in the In-Fusion cloning reaction (Clontech, USA).

Bacterial growth

The derivatives of the eCOMB strain were grown in 56/2 medium containing 2 μg ml^{-1} thymidine at 37°C as described previously (22), with the exception of the *recA441* strain. *recA441* eCOMB cells were mixed with LB medium containing 2 μg ml^{-1} thymidine to give an OD_{600} of 0.02 and grown exponentially to an OD_{600} of 0.3 at 25°C. After being rinsed with M9 salts, the cells were mixed with prewarmed 56/2 medium containing 2 μg ml^{-1} thymidine to give an OD_{600} of 0.1, incubated for 90 min at 42°C, and then treated appropriately for each experiment.

Labelling of cells with radio-labelled thymidine

Cells were mixed with 56/2 medium supplemented with 2 μg ml^{-1} thymidine and [^{14}C] thymidine (0.2 μCi ml^{-1}) as described (22), with minor modifications: the cells were grown exponentially to OD_{600} = 0.3 at 37°C for Δ*lexA* strains and OD_{600} = 0.5 at 42°C for *recA441* strains. At time zero, the pre-labelled cells were rinsed with M9 salts, diluted 3-fold in 56/2 medium supplemented with 2 μg ml^{-1} thymidine and [^{3}H] thymidine (1.0 μCi ml^{-1}), and further incubated at the same temperature. Aliquots (2 ml each) were withdrawn at 5, 10 and 15 min. Radioactivity of ^{3}H and ^{14}C in acid-insoluble material collected by filtration was measured in Emulsifier Scintillator Plus (PerkinElmer) with an AccuFLEX LSC-7200 scintillation counter (Hitachi Aloka Medical, Japan). For normalization in measuring the DNA synthesis rate, the incorporation of [^{3}H] thymidine was divided by that of [^{14}C] thymidine, which is indicative of the initial amount of DNA in cells labelled with [^{3}H] thymidine. The normalized values were plotted at each time point, and slope values were estimated from a linear regression line to determine the DNA synthesis rate. To examine DNA degradation in *recA441* cells during 10 min of incubation, radioactivity of [^{14}C] thymidine in the acid-insoluble fraction of cells collected at 15 min was divided by that at 5 min (Supplementary Figure S3D).

Fork speed determination

To determine the distribution of fork speeds, exponentially growing cells were pulse-labelled with 50 μg ml^{-1} CldU for 2 min and then with 50 μg ml^{-1} IdU for 2 min (Figure 2A). To estimate mean fork speed, CldU-labelled cells were pulse-labelled with 50 μg ml^{-1} IdU for 1, 2, 3 and 4 min (Figure 2B) except Figure 5 (labelling with IdU for 2 and 3 min). The DNA fibre molecules from the cells were processed with a DNA combing method as previously described (22,35). Labelled regions in the extended chromosomal DNA fibres on glass coverslips were detected by immunostaining with two anti-BrdU antibodies, B44 and BU1/75, as previously described (22). Immunocomplexes were visualized using an Axiovert 200M fluorescence microscope (Zeiss, Germany) with a 63× objective and appropriate filters. The length of IdU tracks on combed CldU-IdU DNA was measured to calculate fork speed. In time-course experiments, the median value of more than 100 measurements at each time point was used to calculate slope by linear regression when the medians were plotted as a function of the IdU-labelling time (Supplementary Figures S2–S6). Mean speed values were determined using the slope values from three independent time-course experiments, except in Supplementary Figure S2A and B (a single experiment) and S5A and B (two independent experiments).

Quantitative western blot analysis

Total cellular proteins were loaded in each lane of an SDS-polyacrylamide gel and separated by gel electrophoresis (26). The resolved proteins were transferred to nitrocellulose membranes (Schleicher and Schuell, Germany) and probed with rabbit anti-RecA or anti-DinB antibodies as described (36). Anti-DinB antiserum was mixed with cell extracts of a Δ*dinB* strain to remove non-specific antibodies (24). Immunoblots were developed with enhanced chemiluminescence reagents (GE Healthcare, USA) to visualize DinB and RecA proteins using an LAS-4000 Mini luminescence image analyzer (GE Healthcare). The linear range for the RecA and DinB protein signals from SOS-constitutive SMR7623 (*lexA51*(Def)) cells (31) was established by serial dilution, and the relative amounts of RecA and DinB in sample cells were determined by comparison with their SOS-induced level in SMR7623.

Measurement of the SOS response

Induction of the SOS response in cells carrying *sulA*::*lacZ′YA*::*kan* was measured by determining the specific activity of β-galactosidase (Miller units) with ONPG as described (27). For quantification of the SOS-induced levels, the amounts of cellular RecA were also monitored by western blotting with anti-RecA antibodies as described above.

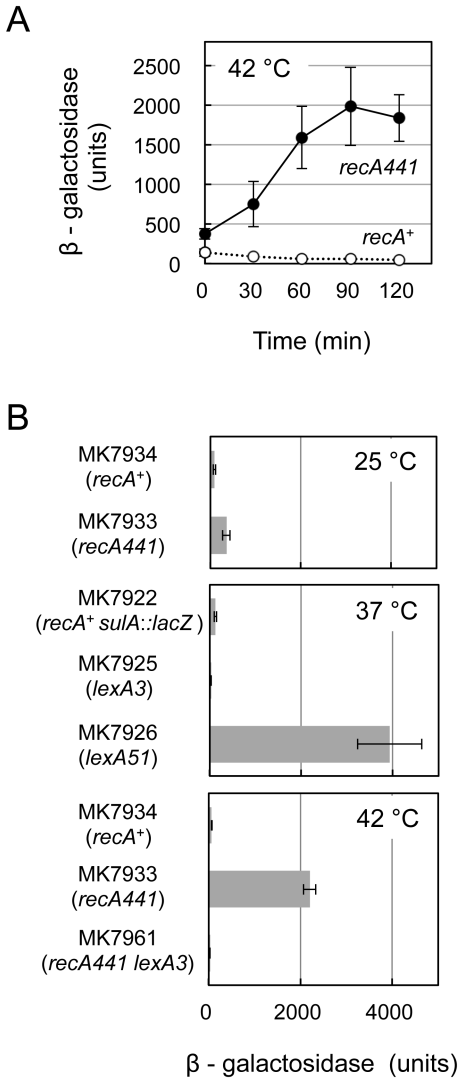

Figure 1. The SOS response of *recA441* cells incubated at various temperatures. Cells were grown exponentially at 25°C and then incubated at the indicated temperatures. The SOS response was examined by measuring the β-galactosidase reporter activity (Miller units) of the *sulA*::*lacZ* chromosomal gene. Error bars are standard errors of the mean (SEMs) for data from more than three independent experiments; some errors were too small to visualize. (**A**) The temperature of the culture was raised to 42°C at time zero, and aliquots were withdrawn at the indicated times. MK7934 (*recA*$^+$) cells, open circles; MK7933 (*recA441*) cells, filled circles. (**B**) Exponentially growing cells were incubated at 25°C (top), 37°C (middle) and 42°C (bottom) for 90 min. Strains are MK7922 (eCOMB *sulA*::*lacZ*), MK7925 (*lexA3 sulA*::*lacZ*), MK7926 (*lexA51 sulA*::*lacZ*), MK7933 (*recA441 srl300*::Tn*10 sulA*::*lacZ*), MK7934 (*srl300*::Tn*10 sulA*::*lacZ*) and MK7961 (*recA441 srl300*::Tn*10 lexA3 sulA*::*lacZ*). MK7933, MK7934 and MK7961 are derivatives of MK7922.

Statistical analysis

An F-test was carried out with Excel (Microsoft, USA) to evaluate if the variations of two independent samples were equal. A two-tailed Student's *t*-test was performed with Excel to evaluate the difference of the mean values of the two independent samples. Asterisks indicate statistically significant difference: $*P < 0.05$ and $**P < 0.005$. A value of $P > 0.05$ was considered not significant (NS).

RESULTS

The checkpoint-dependent delay of replication fork progression has been investigated in eukaryotes using exogenous DNA-damaging agents (3–7). This was inappropriate for assessing DNA replication fork dynamics in the *E. coli* SOS response, because DNA synthesis can be passively inhibited shortly after DNA damage due to the approximately 10-fold faster rate of replication fork progression on the much smaller chromosome of *E. coli* than on those of eukaryotes. To avoid this problem, we took advantage of the thermo-sensitive co-protease allele *recA441* (formerly named *tif-1*) (37). The *recA441* strain induces RecA*-mediated SOS expression at 42°C, but not below 30°C, in the absence of exogenous DNA damage.

Replication fork speed globally decreases in *recA441* cells undergoing physiological SOS induction

We introduced the *recA441* allele into eCOMB having *sulA*::*lacZ* (MK7922 in Supplementary Table S1), which both served as a β-galactosidase reporter for SOS induction and circumvented cell division arrest caused by SulA under the SOS-induced condition. We confirmed that replication fork speed was unaffected by the introduction of Δ*sulA* into the eCOMB strain (Supplementary Figure S1A). The resulting mutant *recA441* (MK7933) and control *recA*$^+$ (MK7934) strains were pre-cultured at 25°C to largely suppress SOS (Figure 1). The SOS response was induced maximally in the *recA441* cells 90 min after the temperature shift to 42°C (Figure 1A), but only reached 56% of the fully SOS-induced level of *lexA51* (Def) cells (Figure 1B). To examine the distribution of individual fork speeds under SOS induction, the cells were incubated further at 42°C sequentially with CldU for 2 min and IdU for 2 min to pulse-label newly synthesized DNA (Figure 2A) (22). CldU- and IdU-labelled regions in the extended chromosomal DNA fibres on glass coverslips were detected by immunostaining, and the lengths of IdU tracks that were adjacent to CldU tracks were measured to determine the speed of individual ongoing replication forks (22). The majority of the forks in the *recA441* cells (79%) moved within the range from 350 to 600 nt s^{-1}, which is considerably slower than the 600−800 nt s^{-1} range observed for most (70%) of the forks in the *recA*$^+$ cells incubated under identical conditions (Figure 2C). The slow fork movement was not attributable to DNA degradation during the labelling period (Supplementary Figure S3D). These results show that replication fork progression was globally retarded when the *recA441* cells underwent a physiological SOS response at the higher temperature.

SOS induction is required for slowdown of fork progression in *recA441* cells

To accurately estimate the mean fork speed, newly synthesized DNA in the cells was labelled with IdU in 1-min increments for 4 min after CldU labelling for 2 min at 42°C (Figure 2B) (22). The median length of IdU-labelled DNA at each time point was plotted as a function of time and analysed by linear regression in three independent experiments (Supplementary Figure S3A–C) (22). The mean fork

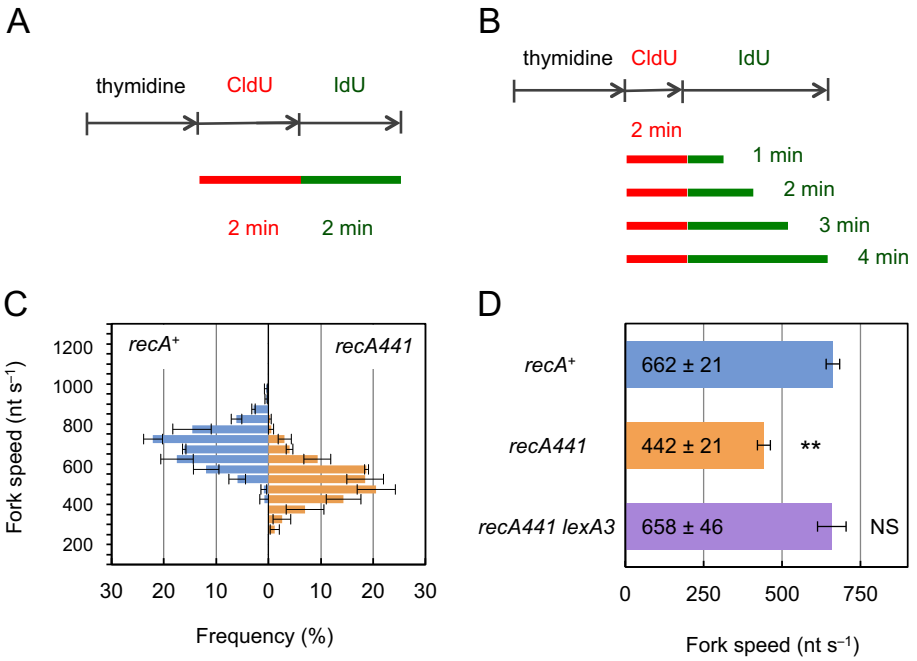

Figure 2. Reduced fork speed in *recA441* cells inducing the SOS response. (**A, B**) Diagrams of DNA labelling. Cells were grown exponentially in 56/2 medium containing 2 μg ml^{-1} thymidine. The cells were pulse-labelled in 56/2 medium lacking thymidine with 50 μg ml^{-1} CldU (red) for 2 min followed by 50 μg ml^{-1} IdU (green) (A) for 2 min and (B) for 1, 2, 3 and 4 min. To determine the speed of ongoing replication forks, the DNA fibres observed were dual-labelled CldU-IdU molecules. (C, D) Fork speed. eCOMB cells were grown exponentially at 42°C. Blue, yellow and purple bars represent MK7934 (*recA*$^{+}$), MK7933 (*recA441*) and MK7961 (*recA441 lexA3* (Ind^{-})), respectively. All strains carry *sulA*::*lacZ′YZ* and *srl300*::Tn*10*. Error bars are SEMs for data from three independent experiments. (**C**) Distribution of fork speeds. Cells were pulse-labelled sequentially with CldU and IdU at 42°C as shown in (A). The total number of DNA fibres observed was 442 for MK7934 (left) and 490 for MK7933 (right). (**D**) Mean fork speed. Cells were pulse-labelled with CldU, followed by IdU in a time course, at 42°C as shown in (B). Values are mean ± SEM. The number of DNA fibres observed in each time-course experiment is shown in Supplementary Figures S3 and S6. The statistical significance of differences between test cells and MK7934 control cells was evaluated by two-tailed Student's *t*-test: $^{**}P < 0.005$; NS, not significant ($P > 0.05$).

speed was determined from three slope values to be 442 ± 21 nt s^{-1} in the *recA441* cells and 662 ± 21 nt s^{-1} in the control cells (mean ± SEM) (Figure 2D), indicating that the forks in the former cells proceed at 67% ($P = 0.0017$) of their rate in the latter cells. In contrast, fork speed in both cells was comparable at the permissive temperature of 25°C, albeit slower than at 42°C (Supplementary Figure S2A and B). The fork speed in eCOMB was reduced with decreasing temperature (Supplementary Figure S2C), suggesting that pacemaking of fork progression by Pol III may be temperature-dependent *in vivo* (22). When the SOS response was suppressed in *recA441* cells by the SOS-noninducible *lexA3* (Ind^{-}) mutation at 42°C (MK7961, Figure 1B), the fork speed was almost the same as that in *recA*$^{+}$ cells (Figure 2D). Together, these data show that SOS induction is absolutely required for the reduced rate of replication fork progression in *recA441* cells.

Fork speed is reduced in constitutively SOS-expressing cells

The RecA441 protein is more efficient than wild-type RecA in interacting with SSB-coated ssDNA to form nucleoprotein filaments at 42°C (38). To determine whether the enhanced competition with SSB for ssDNA binding on its own contributed to fork speed reduction in addition to doing so indirectly through SOS induction, DNA replication was examined for the eCOMB Δ*sulA* Δ*lexA* strain (MK7456). The Δ*lexA* cells almost fully expressed the constitutive SOS response in the absence of the LexA repressor at 37°C (Figure 3). As observed for the *recA441* cells, most of the individual forks in the Δ*lexA* cells moved more slowly than those in the control *lexA*$^{+}$ cells (top and middle graphs of Figure 4A). The mean fork speed in the former cells was 347 ± 13 nt s^{-1}, 55% ($P = 1.9 \times 10^{-4}$) of that in the latter cells (Figure 4B and Supplementary Figure S4). When the chromosomal Δ*lexA* mutation of the MK7456 (eCOMB Δ*lexA*) strain was complemented with a pNTR-*lexA* plasmid expressing the *lexA* gene (Figure 3, Supplementary Table S2), fork speed in the Δ*lexA* cells was restored to the control level (Figure 5 and Supplementary Figure S1B). This speed recovery was not observed with pNTR-*thrA* expressing the *thrA* gene. pNTR-*thrA* was used as a control plasmid because cells carrying an empty vector exerted an inhibitory effect on cell growth under our experimental conditions. These results show that constitutive SOS expression by *lexA* inactivation also retards replication fork progression. Therefore, neither the enhanced ssDNA binding of RecA441 nor the activated RecA* filaments were required for reducing fork speed after the onset of the SOS response. To obtain more general insights into fork speed control in the damage response, we next analysed DNA replication in the Δ*lexA* cells at 37°C.

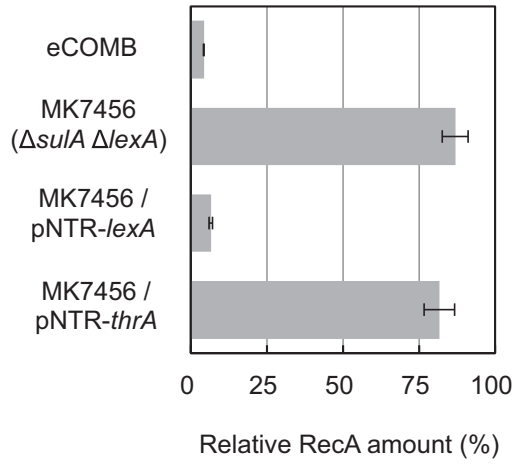

Figure 3. The SOS response in Δ*lexA* cells. MK7456 (Δ*lexA*) cells carrying either pNTR-*thrA* (MK7456/pNTR-*thrA*) or pNTR-*lexA* (MK7456/pNTR-*lexA*) were grown exponentially in 56/2 medium containing 2 μg ml^{-1} thymidine and 0.5 mM IPTG at 37°C. eCOMB and MK7456 cells were also grown exponentially in the same medium but without IPTG at 37°C. Exponentially growing cells were cultured for 6 min in 56/2 medium containing 50 μg ml^{-1} IdU in place of thymidine at 37°C. RecA protein in the total protein fraction of these cells was detected by western blotting with anti-RecA antibodies. The bar graph shows the amount of RecA relative to that in fully SOS-induced SMR7623 (*lexA51*) cells (Supplementary Table S1). Data for eCOMB are the same as those reported previously (22). Error bars are the SEMs for data from three independent experiments.

dinB is required to decrease fork speed in the SOS response

E. coli has three specialized DNA polymerases that dynamically access stalled replication forks and engage slow chain elongation in translesion synthesis (TLS): Pol II, DinB and Pol V (39,40). SOS induction upregulates the genes encoding these DNA polymerases: *polB* for Pol II, *dinB* for DinB and *umuDC* for components of Pol V (after autocleavage of UmuD to UmuD′ facilitated by RecA*) (13). To test the hypothesis that upregulation of these genes might be responsible for the reduced fork speed during SOS induction, Δ*dinB,* Δ*polB* and Δ*umuDC* mutations were introduced individually into the Δ*lexA* cells (MK7456). Deletion of *dinB* boosted slightly but significantly the fork speed of the Δ*lexA* cells ($P = 0.022$; Figure 4B). The increase in the mean fork speed indicates that the physiologically elevated level of DinB causes 28% of the reduction of fork speed in the SOS response. Since neither Δ*polB* nor Δ*umuDC* restored fork speed (Figure 4B and Supplementary Figure S5A), retardation of ongoing fork progression in the SOS response was not a general feature of genes encoding the TLS DNA polymerases. Although upregulation of *umuDC* causes a delayed resumption of DNA synthesis after DNA damage (15), the inhibitory function of *umuDC* on replication forks is probably distinct from that of *dinB*. Since RecA is not activated in the Δ*lexA* cells, we cannot rule out the possibility that Pol V, which is composed of UmuD′$_2$C and RecA*, affects progression of unperturbed forks during the SOS response.

recA also contributes to reduced fork speed in the SOS response

RecA and UvrD act at stalled replication forks (41,42) and are the two most abundant proteins among the SOS gene products (43). To identify other gene(s) responsible for the slowdown of fork speed in Δ*lexA* cells, we examined fork speed in cells lacking either *recA* or *uvrD*. Δ*recA* accelerated the fork speed in the Δ*lexA* cells by 1.5-fold ($P = 0.0031$) (Figure 4B), meaning that RecA accounts for 63% of the reduction of fork speed in the SOS response. In contrast, the reduced fork speed in Δ*lexA* cells was not accelerated by the introduction of Δ*uvrD* (Supplementary Figure S5B).

There is an intimate relationship between homologous recombination (HR) and DNA replication to ensure replication fork progression in both *E. coli* (41,44) and eukaryotes (45–47). A *lexA*-defective *E. coli* strain shows a hyper-recombination phenotype, presumably due to the transcriptional induction of numerous recombination genes including *recA*, which plays a pivotal role in HR (48). To examine the effect of the RecA-mediated HR reaction on fork progression, fork speed was determined in Δ*lexA* cells deficient in two genes that are involved in HR: *recO* for loading RecA onto SSB-coated gapped DNA, and *ruvA* for processing Holliday junctions, the DNA intermediate in the HR reaction. The decreased fork speed in the Δ*lexA* cells was largely unaffected in both Δ*recO* and Δ*ruvA* cells (Figure 4B), indicating that the *recA*-mediated slowdown was not attributable either to RecA loading onto ssDNA mediated by RecFOR or to HR reactions catalyzed by RecA at ongoing replication forks. The small increase of the fork speed in the Δ*recO* cells was statistically insignificant ($P = 0.12$). We cannot exclude the possibility that the fork-control function of RecA needs the other mediator, RecBCD, which loads RecA onto ssDNA that is created at a double-stranded DNA break (DSB), although DSBs are generated following the collapse or stall of replication forks (49).

recA and *dinB* play independent roles in the slowdown of fork progression during the SOS response

The genetic relationship between *dinB* and *recA* in the SOS-dependent reduction of fork speed was studied using Δ*lexA* cells lacking both genes. The distribution profile of fork speeds in the cells was similar to that in *lexA*$^+$ cells (Figure 4A), and the mean speed, 633 ± 18 nt s^{-1}, was statistically indistinguishable from that in eCOMB cells (Figure 4B and Supplementary Figure S5C–F). This is in good agreement with the recovery (91%, the sum of 28% and 63%) of fork speed in Δ*lexA* cells that would be expected if *dinB* and *recA* contributed additively to reduce fork speed. Thus, although RecA can physically and functionally interact with DinB (32,50–53), *dinB* and *recA* were independently responsible for one-third and two-thirds, respectively, of the entire reduction of fork speed observed in cells expressing the SOS response. This implies that at least two distinct regulatory mechanisms modulate replication fork progression in the SOS response.

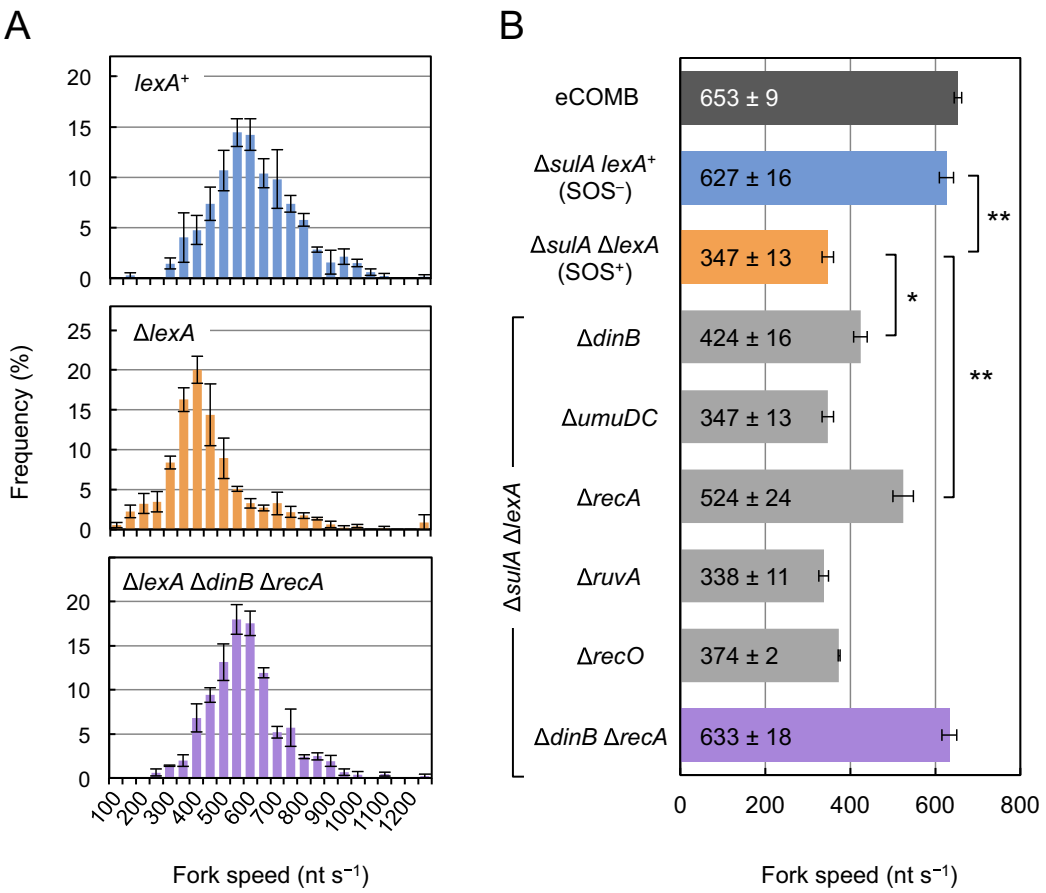

Figure 4. Reduction of fork speed by DinB and RecA in SOS-expressing Δ*lexA* cells. Cells were grown exponentially in 56/2 medium containing thymidine at 37°C. All strains except eCOMB carry Δ*sulA*. Blue, yellow, purple and dark grey represent MK7452 (*lexA*$^+$), MK7456 (Δ*lexA*), MK7498 (Δ*lexA* Δ*dinB* Δ*recA*) and eCOMB, respectively. Light grey bars show MK7460 (Δ*lexA* Δ*dinB*), MK7466 (Δ*lexA* Δ*umuDC*), MK7486 (Δ*lexA* Δ*recA*), MK7916 (Δ*lexA* Δ*ruvA*) and MK7954 (Δ*lexA* Δ*recO*). (**A**) Distribution of fork speeds. Cells were pulse-labelled sequentially with CldU and IdU at 37°C as shown in Figure 2A. The number of DNA fibres observed was 448 for MK7452 (top), 528 for MK7456 (middle) and 496 for MK7498 (bottom). (**B**) Mean fork speed. Cells were pulse-labelled sequentially with CldU and IdU at 37°C as shown in Figure 2B. The value for eCOMB was previously reported by Pham *et al.* (22); the other speed data were determined from the slope values shown in Supplementary Figures S4–S6. The number of DNA fibres observed in each time-course experiment is also shown in Supplementary Figures S4–S6. Error bars indicate the SEMs from three independent experiments. *$P < 0.05$; **$P < 0.005$ (Student's *t*-test).

The rate of overall DNA synthesis is reduced in cells expressing the SOS response

To examine the effect of reduced fork speed on DNA replication in cells expressing the SOS response, initial rates of nucleotide incorporation (within 15 min) into the cells were measured with [^{3}H] thymidine. The *recA441* cells incorporated [^{3}H] thymidine at a rate 15% lower than that in the control *recA*$^+$ cells at 42°C ($P = 0.019$, Figure 6A). Similarly, thymidine incorporation was decreased by 28% in Δ*lexA* cells compared with *lexA*$^+$ cells at 37°C ($P = 0.0046$, Figure 6B). Moreover, the introduction of *lexA3* (Ind$^-$) into the *recA441* cells and the deletion of both *dinB* and *recA* in the Δ*lexA* cells restored the reduced rate of DNA replication to the level observed in the corresponding control cells (Figure 6). Therefore, the SOS response restricted DNA replication by negatively regulating fork progression.

Exclusive upregulation of *dinB* and *recA* inhibits replication fork progression

To investigate whether co-upregulation of the other SOS genes is required for slowing fork speed mediated by *dinB* and *recA*, we overexpressed either *dinB* or *recA* at the SOS-induced levels in non-stressed eCOMB *sulA*::*lacZ* (MK7922) cells. This was achieved by construction of the single-copy *dinBo* and *recAo* plasmids (Supplementary Table S2), which have operator-constitutive mutations in the predicted LexA binding sites of both genes to alleviate the transcriptional repression by LexA. SOS-uninduced cells carrying the *dinBo* and *recAo* plasmids contained DinB and RecA, respectively, at levels almost the same as those in constitutively SOS-expressing *lexA51* (Def) cells (Figure 7A and B). The amounts of intracellular proteins in the *lexA51* (Def) cells were 9-fold higher for DinB and 18-fold higher for RecA than those in MK7922 cells carrying

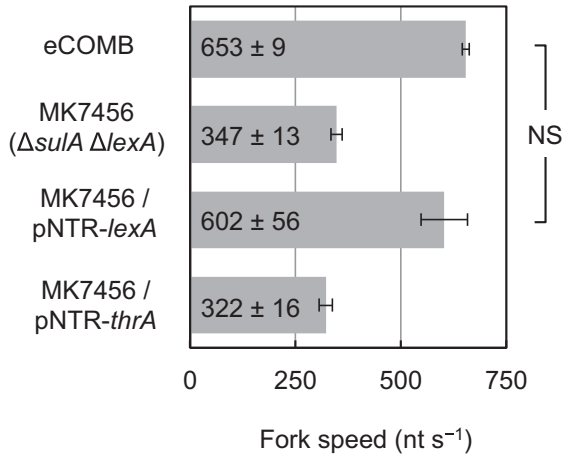

Figure 5. Normal fork speed in Δ*lexA* cells carrying a *lexA*$^+$ plasmid. Cells were grown exponentially as described in the legend of Figure 3. MK7456/pNTR-*thrA* and MK7456/pNTR-*lexA* cells were pulse-labelled in 56/2 medium lacking thymidine with 50 μg ml^{-1} CldU for 2 min and then with 50 μg ml^{-1} IdU for either 2 or 3 min at 37°C, in triplicate. Fork speed was calculated from the difference between the median values of IdU track length in CldU-IdU DNA determined at each time point as shown in Supplementary Figure S1B. The number of DNA fibres observed in each experiment is also shown in Supplementary Figure S1B. The fork speed values for eCOMB and MK7456 are the same data as displayed in Figure 4B. Error bars are the SEMs for data from three independent experiments. NS: not significant ($P > 0.05$, Student's *t*-test).

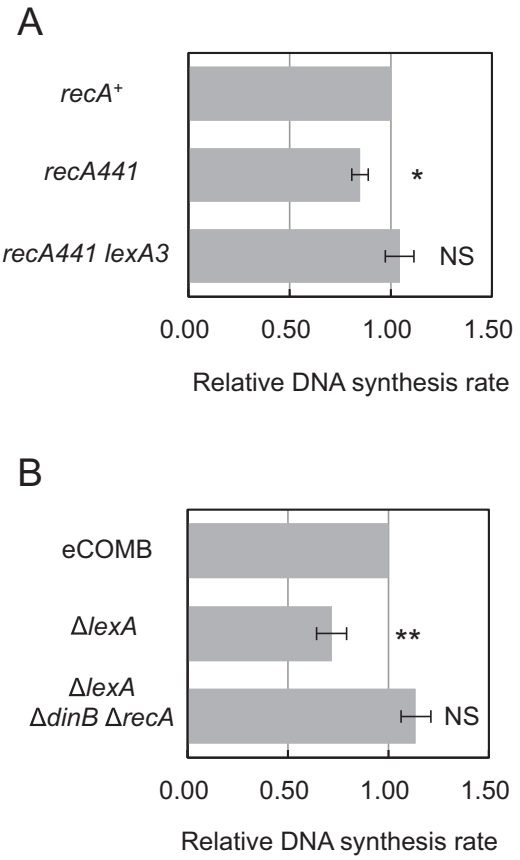

Figure 6. Reduced rate of DNA synthesis in SOS-expressing cells. Cells pre-labelled with [^{14}C] thymidine were incubated in 56/2 medium containing [^{3}H] thymidine for the indicated time at (A) 42°C and (B) 37°C. The ratio of [^{3}H] thymidine to [^{14}C] thymidine incorporated is expressed relative to that of the control cells. Cells were (**A**) control MK7934 (*recA*$^+$), MK7933 (*recA441*) and MK7961 (*recA441 lexA3*), and (**B**) control eCOMB, MK7456 (Δ*lexA*) and MK7498 (Δ*lexA* Δ*dinB* Δ*recA*). Error bars indicate the SEMs from three independent experiments (four for MK7456). Statistically significant differences were evaluated between test cells and the control cells. *$P < 0.05$; **$P < 0.005$; NS: not significant ($P > 0.05$, Student's *t*-test).

empty vector (Figure 7A and B), values which are similar to those previously reported (43,54). The solo upregulation of *dinB* in cells carrying the *dinBo* plasmid reduced fork speed to 70% of that in cells carrying the empty vector plasmid (Figure 7D); a similar result (71%) was obtained for the *recA*-overexpressing cells. As previously reported (21,55), eCOMB cells with excess RecA or DinB did not induce the SOS response under our experimental conditions (Figure 7C). Since replication fork failure, in addition to DNA damage, induces the SOS response (1,9), this lack of SOS induction also suggests that the slowing of fork progression by DinB and RecA is not detrimental to fork stability. Taken together, these results ruled out any additional requirement for the upregulation of other SOS genes to achieve slowdown of fork speed, including those downstream of the *dinB* and *recA* operons, *yafNOP* and *recX,* respectively. Fork speed retardation therefore requires upregulation of only *dinB* and *recA* among the SOS genes.

DISCUSSION

The SOS response of *E. coli* was the first DNA repair network to be discovered (56), and 40 years of subsequent research have revealed a coordinated inducible cellular reaction to DNA damage in bacteria. Here, using DNA fibre analysis in our newly developed eCOMB strain (22), we found that the SOS response actively decreases the rate of ongoing replication fork progression by upregulating *dinB* and *recA*. This new role for both genes in the SOS response implies that the specialized DNA polymerase, DinB, and the main recombinase, RecA, can gain access to active replication forks and thereby control replication dynamics under replication stress. Whereas the leftward and rightward replication forks are independent in *E. coli* (57,58), the SOS response may provoke functional crosstalk between stalled and moving forks through these proteins to maintain genome integrity under replication stress.

Inducible stable DNA replication (iSDR) is an alternative and DnaA-independent replication mode that is activated to recover DNA synthesis under SOS-inducing conditions in the absence of concomitant protein synthesis (59). RecA* is needed to induce iSDR even after SOS expression (60). Since our analyses of fork speed were performed in Δ*lexA* cells without RecA* (Figures 4 and 5), the observed reduction in fork speed involved replication forks in the normal *oriC* replication system rather than in iSDR. During normal DNA replication, a rapidly growing *E. coli* cell has two to eight replication forks (61). Our findings suggest that when a DNA lesion inhibits a replication fork, the SOS response restricts progression of the remaining forks (Figures 2 and 4). This control strategy could, for example, conserve the lim-

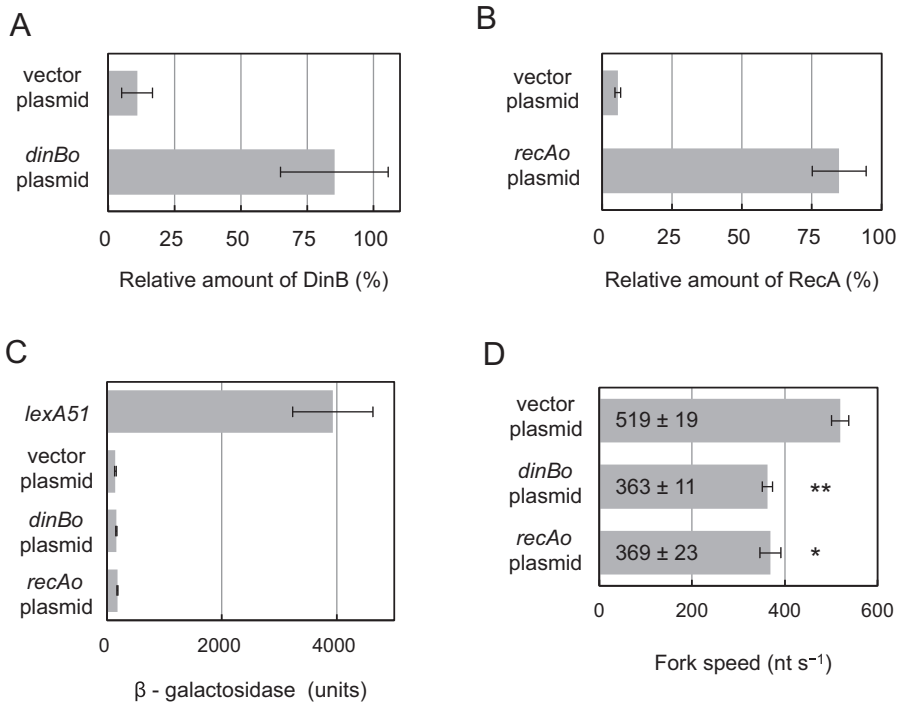

Figure 7. Slowdown of fork speed in cells overexpressing *dinB* or *recA*. Cells were grown exponentially in 56/2 medium containing thymidine at 37°C. (A, B) Amounts of proteins overproduced. (**A**) DinB and (**B**) RecA were detected in the total protein fraction of MK7922 (*lexA*$^+$ *dinB*$^+$ *recA*$^+$) cells carrying *dinBo* plasmid pSCG3 and *recAo* plasmid pSCG11, respectively, by western blotting with appropriate antibodies. The empty plasmid vector pCC1BAC served as a control. The graphs show the amount of each protein relative to that in fully SOS-induced *lexA51* cells (SMR7623). (**C**) SOS response. The specific activity of β-galactosidase (Miller units) was determined with the *sulA-lacZ* fusion reporter gene on the chromosome of MK7922 carrying each plasmid and MK7926 (*lexA51*). (**D**) Mean fork speed. Cells were pulse-labelled with CldU followed by IdU at 37°C as shown in Figure 2B, and mean fork speed was determined with the slope values shown in Supplementary Figure S6. Error bars indicate the SEMs from three independent experiments. The number of DNA fibres observed in each time-course experiment is shown in Supplementary Figure S6. Statistically significant differences compared with pCC1BAC (Student's *t*-test): $^{**}P = 0.0019$ for pSCG3 and $^{*}P = 0.007$ for pSCG11.

ited pool of nucleotides for repair DNA synthesis (62) and provide time for the cell to repair other lesions before they are encountered by unperturbed replication forks. Alternatively, the slow progression of replication forks could reduce potential conflicts between the transcription machinery and the replisome when the SOS response triggers massive induction of many SOS genes (63).

The 30–50% decrease in fork speed in the SOS response (Figures 2 and 4) did not correspond precisely with the magnitude of the reduction in bulk DNA synthesis (20–30%; Figure 6). The reduction of fork speed may be partially compensated for in total DNA replication, perhaps by an increase in the number of slow forks through the overlapping of replication cycles, in which new DNA replication initiates before the previous round is completed. Alternatively, this difference may be the result of our using two distinct experimental approaches: one value arises from summing the speeds of individual forks, and the other from directly analyzing overall DNA synthesis in a large bulk population. In any case, slow fork progression retards DNA replication in the SOS response. However, it remains unknown if the slow DNA replication in the bacterial SOS response functions to avoid genetic instability, as occurs in the eukaryotic intra-S-phase checkpoint. To address this in further studies, we need to isolate and study *dinB* and *recA* mutations that separate the abilities of the proteins to decrease fork speed from their known activities in DNA damage tolerance. Nevertheless, unlike the intra-S-phase checkpoint, which does not arrest cell cycle progression, the SOS response in wild-type cells rapidly blocks bacterial cell division through induction of the *sulA* gene so that a damaged chromosome can be repaired before being segregated (1). This raises the possibility that fork speed is slowed not for the purpose of delaying DNA replication, as with the intra-S-phase checkpoint, but merely as a consequence of the need to confer robustness on replication fork progression. DinB and RecA rescue replication forks stalled by DNA damage through the universal mechanisms of TLS and HR, respectively (1). HR proteins are required to ensure fork progression by protecting newly replicated DNA from resection at fork obstacles (45). DinB and RecA proteins may be recruited to moving forks during the damage response in anticipation of fork blockage at DNA lesions ahead. As a secondary consequence of such a damage-tolerance function, activated by these proteins, the fork may move slowly (64).

Our data (Figures 4 and 7D) provide supporting evidence for our model that DinB acts as a brake to modulate fork progression in the DNA damage response (20). DinB takes over DNA synthesis from Pol III at a primer-template junction in a dose-dependent manner and synthesizes DNA chains much more slowly than Pol III does *in vitro* (16–19). Structural requirements of DinB for the *in vitro* inhibition

of a moving Pol III match those of replication inhibition by ectopic *dinB* overexpression (20). Thus, it is very likely that DinB, at the increased level seen in SOS induction, directly interrupts progression of replication forks by replacing Pol III* on the β clamp of the replisome and catalyzing slow chain elongation by forming an alternative replisome with DnaB helicase in place of Pol III* (16–20). In this scenario, DinB can also dislodge Pol III* from the β clamp on the lagging strand and slowly elongate the DNA chain of Okazaki fragments. This inhibition of Pol III probably leads to an accumulation of SSB-coated ssDNA gap regions, which are the inducing signals of the SOS response. However, upregulation of *dinB* does not significantly induce the SOS response in the presence of *recFOR* (Figure 7C) (21). Since DNA synthesis continues at a reduced rate at slowly moving forks, unlike at stalled forks, the gap may not persist for long enough to allow RecA to initiate nucleation for nucleoprotein filament formation.

Pol II, as well as DinB, can, at high levels, displace Pol III* from the β clamp on a template DNA *in vitro* (18,65). Uncleaved UmuD and UmuC play a role in regulating DNA replication after UV irradiation (15). However, neither Δ*polB* nor Δ*umuDC* restores the slow fork speed in the SOS response (Figure 4B and Supplementary Figure S5A). Replacement of the moving Pol III with DinB is probably more effective in cells expressing the SOS response than replacement with Pol II or UmuDC. DinB is the only translesion Y-family DNA polymerase conserved among bacteria, archaea and eukaryotes (66). Interestingly, aberrant recruitment of the eukaryotic orthologue of DinB, Pol κ, to replication forks reduces replication fork speed in human cells (67). Moreover, a moderate ectopic overproduction of Pol κ slows down replication fork progression (68). It is thus tempting to speculate that Pol κ acts as a checkpoint effector that retards fork progression using a similar mechanism to that of DinB.

Fork speed in *lexA*$^+$ (MK7922) cells carrying the *dinBo* and *recAo* plasmids was reduced to 70% and 71%, respectively, of that in cells carrying the empty vector (Figure 7D). Consistent with the value for the *recAo* plasmid, the fork speed in Δ*dinB* Δ*lexA* cells, which upregulate *recA* but not *dinB* in the SOS response, was reduced to 68% of that in *lexA*$^+$ (MK7452) cells (Figure 4B). Thus, it is unlikely that RecA competes with other proteins for the fork-slowing activity specifically during the SOS response. In contrast, the value for the *dinBo* plasmid was smaller than the relative reduction of fork speed (84%) in Δ*recA* Δ*lexA* cells upregulating *dinB* but not *recA* in the SOS response (Figure 4B). This suggests that during SOS induction, other proteins partially counteract the fork-brake function of DinB. Interstingly, both Δ*polB* and Δ*uvrD* display a slight tendency to reduce fork speed in Δ*lexA* cells (Supplementary Figure S5A and B). Pol II and UvrD together at the induced level in the SOS response may mitigate the brake action of DinB on unperturbed fork progression to minimize untargeted mutagenesis (69). It would also be intriguing to examine whether proteins physically bound to DinB modulate its fork-brake ability through protein–protein interaction (52,70,71).

RecA recombinase, as well as DinB, is highly conserved in all organisms. In damage tolerance, RecA has multiple functions including the rescue of stalled or collapsed replication forks in *E. coli* (1,12). The experiments reported here reveal a new function for RecA: slowdown of fork progression in the SOS response (Figures 4 and 7D). Since it has been suggested that the HR protein modulates replication fork progression on damaged DNA in eukaryotes (46,47), the eukaryotic RecA orthologue Rad51 may slow fork progression in a manner comparable to the SOS response. How does RecA inhibit the movement of unperturbed forks during SOS induction? That *recO* is not required for the reduction of fork speed (Figure 4B) indicates that RecA controls fork progression without the assistance of the RecFOR mediator, which facilitates the loading of RecA onto an SSB-coated ssDNA gap (12). Heat stabilization of RecA441 nucleofilaments was not sufficient to reduce fork speed in SOS-uninducible *lexA3* (Ind$^-$) cells (Figure 2D). *recA*-overexpressing cells having slow forks did not induce the SOS response (Figure 7C), indicating the absence of RecA filaments that would facilitate LexA self-cleavage. Thus, it is unlikely that RecA at the SOS-induced level self-assembles on ssDNA on the lagging strand and thereby exerts some inhibitory effect on leading strand synthesis (53,72). The other possible mechanism for the slowdown of fork speed by RecA is a direct physical interaction between RecA and components of the replisome, not via ssDNA binding by RecA. Such an interaction may be enabled with increased amounts of RecA in the presence of replication hyper-structures (73). One outcome of the interaction could be a reduction in the physical association between DnaB helicase and the τ subunit of Pol III, which is essential for the normal speed of replication fork movement (74). Further studies are needed to address these possibilities.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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