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Investigating the Mechanisms of Ribonucleotide Excision Repair in *Escherichia coli*

Alexandra Vaisman, **John P. McDonald**, **Stephan Noll**[1], **Donald Huston**, **Gregory Loeb**, **Myron F. Goodman**[2], and **Roger Woodgate**[*]

Laboratory of Genomic Integrity, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892-3371, USA

[1]Gene Bridges GmbH, Im Neuenheimer Feld 584, 69120 Heidelberg, Germany

[2]Department of Biological Sciences and Chemistry, University of Southern California, University Park, Los Angeles, California 90089-2910, USA

Abstract

Low fidelity *Escherichia coli* DNA polymerase V (pol V/UmuD′$_2$C) is best characterized for its ability to perform translesion synthesis (TLS). However, in *recA730 lexA*(Def) strains, the enzyme is expressed under optimal conditions allowing it to compete with the cell's replicase for access to undamaged chromosomal DNA and leads to a substantial increase in spontaneous mutagenesis. We have recently shown that a Y11A substitution in the "steric gate" residue of UmuC reduces both base and sugar selectivity of pol V, but instead of generating an increased number of spontaneous mutations, strains expressing *umuC*_Y11A are poorly mutable *in vivo*. This phenotype is attributed to efficient RNase HII-initiated repair of the misincorporated ribonucleotides that concomitantly removes adjacent misincorporated deoxyribonucleotides. We have utilized the ability of the pol V steric gate mutant to promote incorporation of large numbers of errant ribonucleotides into the *E. coli* genome to investigate the fundamental mechanisms underlying ribonucleotide excision repair (RER). Here, we demonstrate that RER is normally facilitated by DNA polymerase I (pol I) via classical "nick translation". *In vitro*, pol I displaces 1–3 nucleotides of the RNA/DNA hybrid and through its 5′→3′ (exo/endo) nuclease activity releases ribo- and deoxyribonucleotides from DNA. *In vivo*, *umuC*_Y11A-dependent mutagenesis changes significantly in polymerase-deficient, or proofreading-deficient *polA* strains, indicating a pivotal role for pol I in ribonucleotide excision repair (RER). However, there is also considerable redundancy in the RER pathway in *E. coli*. Pol I's strand displacement and FLAP- exo/endonuclease activities can be facilitated by alternate enzymes, while the DNA polymerization step can be assumed by high-fidelity pol III. We conclude that RNase HII and pol I normally act to minimize the genomic instability that is generated through errant ribonucleotide incorporation, but

[*]To whom correspondence should be addressed: Mailing address: 9800 Medical Center Drive, Building C, Rm 320, Bethesda, MD 20892-3371, USA, Tel: +1 301-217-4040; Fax: +1 301-217-5815; woodgate@nih.gov.

Conflict of interest statement

The authors declare that there is no conflict of interest

that the “nick-translation” activities encoded by the single pol I polypeptide can be undertaken by a variety of back-up enzymes.

Keywords

Y-family DNA polymerase; Steric gate mutant; Ribonucleotide excision repair; UmuC; Spontaneous mutagenesis; RNase H

1. Introduction

Y-family DNA polymerases are best characterized for their ability to traverse lesions in DNA that would otherwise block the cell’s replicase. Structural studies of these polymerases indicate spacious active sites that readily accommodate large bulky adducts within their confines [1]. While this is an extremely important characteristic for efficient lesion bypass, it concomitantly reduces base-selection fidelity when the polymerases replicate undamaged DNA. We recently reported that in addition to low-fidelity DNA synthesis, the *E. coli* Y-family polymerase pol V ($UmuD'_2C$) also exhibits poor sugar discrimination and readily incorporates ribonucleotides into DNA *in vitro* [2]. The ability to incorporate ribonucleotides can be modulated by mutations at, or adjacent to, the so-called “steric gate” residue that normally serves to block errant ribonucleotide insertion by DNA polymerases [3]. The steric gate in pol V has been identified as Y11 in the catalytic UmuC subunit of pol V. Not surprisingly, substitution of the large aromatic tyrosine residue by the much smaller aliphatic alanine residue essentially “unlocks” the steric gate and leads to a dramatic increase in the efficiency of pol V-dependent ribonucleotide incorporation [2]. Based upon the tendency of *umuC*_Y11A to efficiently misincorporate both ribo- and deoxyribonucleotides into DNA, we expected cells expressing pol V steric gate mutant to exhibit a mutator phenotype *in vivo*. However, to our surprise, both spontaneous and UV-induced *umuC*_Y11A-dependent mutagenesis were considerably lower than that observed with wild-type pol V [2, 4]. Recently, we have shown that the reduced mutability of the *umuC*_Y11A expressing strain is caused by efficient repair of the errantly incorporated ribonucleotides with the concomitant removal of adjacent misincorporated deoxyribonucleotides [5]. These conclusions were drawn from the observation that the level of *umuC*_Y11A spontaneous mutagenesis increases dramatically in Δ*rnhB* strains lacking endoribonuclease RNase HII [5].

RNase HI cleaves the 3′-O-P bond of the RNA moiety in the RNA/DNA hybrids with more than four sequential rNMPs embedded in a dsDNA strand, while RNase HII can hydrolyze all kinds of hybrids preferring those having a single rNTP over rNTP stretches. Although the role of RNase H enzymes in releasing rNMPs from bacterial DNA has been extensively studied, the precise pathway initiated by these proteins remains to be established. Based on *in vitro* studies, a general model describing the sequence of events leading to the replacement of a ribonucleotide embedded in DNA with deoxyribonucleotide has been proposed for eukaryotic organisms [6–9]. The ribonucleotide excision repair (RER) pathway in eukaryotes is initiated when RNase HII nicks the phosphodiester bond between the dNMP and rNMP. Then, a second cut 3′ to the rNMP is made by the FLAP endonuclease, FEN-1. Following the release of the cleaved mono-ribonucleotide, pol δ fills the resulting gap by

strand displacement DNA synthesis after which DNA ligase seals the nick completing the repair pathway.

As with many other cellular processes, some steps of RER can be accomplished quite effectively by alternative enzymes [9]. For example, a second cut 3′ to rNMP can be made by Exo1 instead of FEN1, while pol ε can substitute for pol δ in the gap-filling step [9]. In contrast, *S. cerevisiae* RNase HII appears to be essential for the successful processing of rNMPs embedded into DNA since deletion of the *RNH201* gene encoding the catalytic subunit of RNase HII leads to replicative stress and genome instability [10]. The instability results from triggering the endoribonuclease activity of topoisomerase 1 (Top1) [11]. In contrast to RNase HII-dependent cleavage, which generates 3′-hydroxyl and 5′-phospho-ribonucleotide ends, Top1-catalyzed cleavage of a bond between ribo- and deoxynucleotides leads to the formation of 2′-3′-cyclic phosphates, which are refractory to religation. As a result, stable ssDNA breaks are formed at the sites of incorporated rNMPs, and when these breaks are located within short repeats, their repair often leads to small deletions and genomic instability [11].

Compared to yeast, bacterial cells appear to be better equipped to withstand errant ribonucleotides inserted into chromosomal DNA. We have shown recently that in the absence of RNase HII, *E coli recA730 lexA*(Def) Δ*dinB* strains grow normally and ribonucleotides incorporated by *umuC*_Y11A can either be removed by nucleotide excision repair (NER), or in a repair pathway initiated by RNase HI [5, 12]. The involvement of RNase HI in RER in this case, is possible because the sugar discrimination of *umuC*_Y11A is so low that it is able to catalyze incorporation of multiple ribonucleotides consecutively producing RNA/DNA hybrids which could be processed by RNase HI [5]. NER, on the other hand, is able to excise isolated rNMPs as well as RNA fragments although less efficiently than RNase HII-dependent RER [12].

The purpose of the current study is to identify the essential components of the prokaryotic RER pathway. To do so, we have taken advantage of the fact that the Y11A steric gate mutant of *E. coli* pol V readily incorporates ribonucleotides into DNA and thereby triggers the onset of RER [2]. We have already established that the main repair process is initiated by RNase HII when it nicks DNA 5′ to the incorporated ribonucleotide [5]. But how is the repair process completed? Assuming that there are similarities between the eukaryotic and prokaryotic pathways, repair of ribonucleotides errantly incorporated into the genome would need the participation of a DNA polymerase that can catalyze strand-displacement at the nick and a FLAP endonuclease to remove the displaced ribo- and deoxyribonucleotides.

The unique enzymatic properties of DNA polymerase I make it an ideal candidate for the job [13, 14]. The pol I polypeptide (103 kDa) encoded by the *polA* gene, consists of two functional domains, a large (68 kDa) C-terminal domain (Klenow fragment) possessing DNA polymerase, strand separation, and 3′→5′ exonuclease activities [15, 16], and a small (35 kDa) N-terminal fragment containing 5′→3′ exo- and FLAP-endonuclease activities [17–20]. The combination of these activities predetermines pol I to be a key component of the *E. coli* replication machinery by playing an essential role during Okazaki fragment processing [21], and fulfilling functions that in eukaryotes requires the coordinated action of

two enzymes, pol δ and FEN1. By means of its 5′→3′ exonuclease activity, pol I removes RNA primers that are used to initiate Okazaki fragment synthesis, while via strand displacement DNA synthesis and 3′→5′ proofreading, it accurately fills the resulting gaps in the lagging strand [22].

Here, we present compelling biochemical and genetic evidence that the same pol I activities are utilized to complete RNase HII-initiated excision of ribonucleotides errantly incorporated into DNA by pol V and as a consequence, it reduces the mutagenic potential of the low-fidelity polymerase. However, similar to eukaryotes, there is also considerable redundancy in the ribonucleotide repair process in *E. coli* and in the absence of pol I, certain functions are readily assumed by back-up enzymes.

2. Materials and methods

2.1 Bacterial strains, plasmids and oligonucleotides

Bacterial strains used in this study are described in Table 1. Where noted, new strains were generated via generalized transduction using P1*vir* [23]. The various *polA* alleles were transduced into the desired background by selecting for the closely linked antibiotic resistance (Tet^R for *polA107*; Cm^R for *polA*_D424A and *polA*_SD^-; Zeo^R for *polA*_ΔC). In the case of *polA107* and *polA*_ΔC, transductants were screened for sensitivity to methylmethane sulfonate (MMS), whereas the *polA*_D424A and *polA*_SD-transductants were screened by colony PCR (see below). Mismatch repair (MMR) defective derivatives (Δ*mutL*::Kan, Δ*mutL::cat* or *mutL218*::Tn*10*) were usually generated last, so as to limit genomic instability associated with defects in mismatch repair. The exception was the *mutD5 zaf13*::Tn*10* allele that was introduced after the Δ*mutL* allele.

Where noted, the following antibiotics were used for selection; Zeocin (25 μg/ml), Kanamycin (50 μg/ml), Tetracycline (15 μg/ml), Chloramphenicol (20 μg/ml), and Spectinomycin (50 μg/ml).

Plasmids used in this study are listed in supplementary Table 1. Oligonucleotides used in this study are listed in supplementary Table 2.

2.2 Detection of the enzymatic activities of pol I important for ribonucleotide repair pathway

Oligonucleotides were synthesized by Lofstrand Laboratories (Gaithersburg, MD) and gel purified prior to use (Table 3). DNA substrates were prepared byannealing a 49-mer DNA template, XAG49, containing an abasic site 27 nucleotides from the 3′ termini with the 32-mer downstream blocker, FLAPU, with a uracil at its 5′-terminus and the 17-mer (SSP1), or 27-mer (SSP1-27) primer that form a nicked DNA duplex, or a flapped structure, respectively (Fig. 1). In order to separate polymerase and nuclease activities of pol I, either the primer (17-mer or 27-mer), or 32-mer blocker oligonucleotide were 5′-P^{32} labeled before hybridization. Annealing was performed at a 1.5-fold excess of unlabeled over P^{32}-labeled oligonucleotides by heating in an annealing buffer (50 mM Tris-HCl (pH 8), 5 mM $MgCl_2$, 50 μg/ml BSA, 1.42 mM 2-mercaptoethanol) for 10 min at 100°C, followed by slow cooling to room temperature. Wild type pol I was purified as described earlier [24]. DNA substrates

(2 nM) were incubated with pol I (4, 16, 63, 250 pM, or 1 nM) at 37°C in reaction mixtures containing 10 mM Tris-HCl, pH 7.9; 50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol and 100 μM dNTPs. Reactions were stopped by the addition of an equivalent amount of gel loading buffer containing 50 mM EDTA, 0.1% xylene cyanol and 0.1% bromophenol blue in 90% formamide. Reaction products were heat-denatured and separated in a 20% polyacrylamide gel containing 8 M urea and then visualized and quantified using a Fuji image analyzer FLA-5100.

2.3 Construction of a polA mutant defective for strand displacement

E. coli MG1655 was aerobically propagated on M9 minimal (M9) medium or on M9 agar plates. Ampicillin (50 μg/ml), Chloramphenicol (15 μg/ml), Tetracycline 3 μg/ml and Zeocin (25 μg/ml) were added as necessary to liquid or solid media for selection. The genome sequence of *E. coli* MG1655 [25] and Artemis software [26] were used for *in silico* strain construction.

An ~1.7kb *Bst*API-*Mfe*I fragment of the *E. coli polA* gene was chemically synthesized by Genscript (Piscataway, NJ). The synthesized fragment differed from the wild-type *polA* sequence at codons Ser769 (AGT→GCA) and F771 (TTC→GCA). These substitutions introduced a novel *Pst*I site into the fragment for ease of subsequent detection and changed the wild-type amino acid to alanine (S769A and F771A respectively). Based upon previous biochemical studies [27] the dual S769A and F77A substitutions should inactivate pol I's ability to promote strand displacement. For simplicity we will refer to this novel allele as *polA*_SD$^-$. The synthesized 1.7kb *Bst*API-*Mfe*I fragment was sub-cloned into the similarly digested pSKpolAint vector [22] to generate pJM993 (Supplementary Table 1).

Recombineering was performed as described by Gene Bridges (www.genebridges.com) with minor modifications. In brief, 1.4 ml Red/ET proficient *E. coli* strains were grown at 30°C to an OD_{600nm} of ~0.3. Transient expression of the pRed/ET-encoded (Supplementary Table 1) *red* genes was induced by adding 50 μl of 10% (w/v) L-arabinose (Sigma) followed by a temperature increase to 37°C. After 40 min incubation, the cells were washed twice with ice cold 10% (v/v) glycerol (AppliChem) and electroporated with 100 ng of DNA in a chilled 1-mm cuvette at 1.35 kV, 10μF, 600ω using an Eppendorf electroporator 2510. Cells were resuspended in 1 ml ice-cold M9 minimal medium and aerobically grown at 37°C for 3h. M9 plates supplemented with appropriate antibiotics were used to select for recombinants.

A two-step Red/ET recombination approach was used to transfer the pJM993 borne *polA* mutations to the genomic *polA* gene (2787 bp) of *E. coli* MG1655 (Supplementary Fig. 1). In the first step, the chromosomal 3′ *polA* region spanning codons 769 to the ochre codon was replaced by a PCR amplified 664 bpZeo ^{R} cassette displaying 50-base pairs homologous to the genomic target region (primer p3 × p4; Supplementary Table 1). Upon the recombineering step, ~550 transformants were selected on M9+zeo plates. No colonies appeared without induction of the Red/ET machinery. Four of the Zeo^R colonies were analyzed and one, MG1655 *polA*_ΔC,that displayed the correct genomic integration of the Zeo^R cassette as revealed by sequencing, was chosen for further study (Table. 1). In the second step (Supplementary Fig. 1), Zeo^R was replaced by *polA*_SD$^-$_*cat*. Therefore, the pJM993 stretch comprising the 3′ region of *polA*_SD$^-$ encompassing the S769A and F771A

substitutions (i.e. *polA* codons 502 to the ochre stop codon) and adjacent *cm*^{R} marker was PCR amplified using primers cp6 and p2 (Supplementary Table 1). The 2279 bp linear *polA*_SD$^-$_*cat* fragment which shares 800 bp (5′) and 50 bp (3′) of terminal sequence homology with the genomic target locus was used to replace the Zeo^R cassette from Red/ET proficient MG1655 *polA*_ΔC by Red/ET recombination. Several independent recombineering approaches yielded two clones that displayed a Cm^R and Zeo^S phenotype characteristic of the successful displacement of the Zeo^R marker from the MG1655 *polA*_ΔC genome. For one clone, the correct insertion of the *polA*-SD$^-$-*cat* cassette which introduces the S769A and F771A codons and reconstructs the full-length *polA* gene in the resulting strain, MG1655 *polA*_SD$^-$ was confirmed by PCR and DNA sequencing (Supplementary Fig. 1). In addition, the MG1655 *polA*_SD$^-$ clones displayed no growth on M9 plates supplemented with Tetracycline or Ampicillin, which confirms the absence of plasmids pRed/ET and pJM993. The *polA*_SD$^-$ allele was subsequently moved to additional strains of *E. coli* via conventional P1*vir* transduction [23].

2.4 Colony PCR assay to test for polA_SD$^-$ and polA_D424A genotypes

To identify strains carrying the *polA*_SD$^-$ allele, chloramphenicol resistant colonies were subject to colony PCR using primers polA-RBamHI and polA_F2105 (Table 3). Briefly, cells were heated to 95°C for 5 min to lyse cells. An ~700 bp fragment of the *polA* gene was amplified after 40 PCR cycles (95°C for 30sec, 60°C 45sec and 72°C 2min). The ~700 bp amplicon was then digested with PstI. Strains carrying the *polA*_SD$^-$ allele gave products of 225 bp and 478 bp. The *polA_D424A* allele was similarly identified by colony PCR as previously described [22], using primers polA-FD424 and polA-RD424 that yields an amplicon of ~1.2kb (Supplementary Table 2). The *polA*_D424A substitution generates a novel *Bss*HII site in *polA*. The ~1.2 kb amplicon was therefore digested with *Bss*HII and the *polA*_D424A allele confirmed by the appearance of 646 bp and 574 bp fragments.

2.5 Quantitative analysis of spontaneous reversion of the hisG4(oc)

Cells transformed with the low-copy-number empty vector plasmid, pGB2, or pGB2-derived plasmid pRW134, or pJM963, expressing wild-type pol V or *umuC*_Y11A variant respectively (Supplementary Table 1), were grown overnight at 37°C in LB media plus spectinomycin. At least three independent cultures were assayed for each strain. To determine the number of spontaneously arising histidine mutants on the plate, cells were seeded on the Davis and Mingioli minimal agar plates [28] plus glucose (0.4% wt/vol); agar (1.0% wt/vol); proline, threonine, valine, leucine, and isoleucine (all at 100 μg/ml); thiamine (0.25 μg/ml); and either no histidine, 1 μg/ml histidine, or 4μg/ml casamino acids. On the plates lacking histidine, only pre-existing His$^+$ mutants in the culture grew to form colonies. However, on the plates containing 1μg/ml histidine, or 4μg/ml casamino acids, >4 × 10^7 auxotropic bacteria grew to form a lawn, concomitantly exhausting the low level of histidine after 1–2 days incubation. Spontaneously arising His$^+$ mutants grew up through the lawn and were counted after 4 days incubation at 37°C.

We would like to emphasize that the number of spontaneous revertants arising on the plate is a characteristic of the strain assayed. The number of revertants that arise spontaneously during the 4-day incubation is independent of the initial number of bacterial cells plated

(within the range of 10^5 to 10^8 cells [29], but is, instead, dependent on the final number of auxotrophs on the plate and that number is a function of the histidine concentration [30, 31]. The actual number of auxotrophic cells (background lawn) on the plate are unimportant, but are nevertheless assumed to be invariant between the strains assayed, since the histidine, or casamino acid concentration is constant and supports the growth of the same absolute number of auxotropic cells per plate, despite any differences in overall growth rates. As a consequence, the extent of spontaneous mutagenesis is expressed as a frequency (mutants per plate) rather than a rate (mutants per cell plated).

The number of His^+ mutants spontaneously arising on the plate (at least 3 plates for each culture) was determined by subtracting the number of pre-existing mutants that grew on plates lacking histidine. The values reported in the tables shown in Figs. 2, 3 & 5 represent the average number of His^+ mutants per plate from at least 3 independent experiments (± standard error of the mean [SEM]). The *relative* contribution of *umuC*_Y11A mutations was calculated by subtracting the number of mutants arising on plates containing the control vector, pGB2 (and lacking pol V) and then expressing the corrected number of *umuC*_Y11A-dependent His^+ mutants (pJM963), as a percentage of the corrected number of wild-type pol V-dependent His^+ mutants (pRW134). The data plotted in Figs 2, 3 & 5 represent the mean value for the relative *umuC*_Y11A-dependent mutagenesis (± standard error of the mean [SEM]). The differences between these values for various strains were assessed for statistical significance using the Student's t-test (SigmaPlot 11.0; Systat Software Inc., Chicago, IL).

2.6. Effect of polA alleles deficient in 3′→5′ exonuclease activity, or DNA polymerase activity on the spectra of spontaneous mutations observed in the rpoB gene of lexA(Def) recA730 ΔdinB ΔumuDC rnhB$^{+/-}$ strains expressing umuC_Y11A

The mutation spectra were generated using the *rpoB*/Rif^R mutagenesis assay [32, 33]. *E. coli* strains RW1048 [relevant genotype: *lexA*(Def) *recA730* Δ*dinB*Δ *umuDCpolA* _D424A Δ*mutL*], RW1320 [relevant genotype: *lexA*(Def) *recA730* Δ*dinB*Δ *umuDCpolA* _D424A Δ*rnhB*Δ *mutL*], RW1098 [relevant genotype: *lexA*(Def) *recA730* Δ*dinB*Δ *umuDC*Δ *polA*_ΔC Δ*mutL*], and RW1336 [relevant genotype: *lexA*(Def) *recA730* Δ*dinB*Δ *umuDC*Δ *polA*_ΔC Δ*rnhB* Δ*mutL*], expressing the *umuC*_Y11A plasmid (pJM963) were diluted from frozen stocks and the cultures containing <1000 viable cells were grown in LB for at 37°C. The next day, appropriate dilutions were spread on an LB agar plates containing 100 μg/ml rifampicin. Individual independent Rif^R colonies were subjected to PCR in a 96-well micro-titer plate using the PCR primers RpoB1: 5′-CAC ACG GCA TCT GGT TGA TAC AG-3′ and RpoF1: 5′-TGG CGA AAT GGC GGA AAA C-3. An <1 kb central region of the *rpoB* gene was amplified by denaturation at 95°C for 3 min, followed by 30 cycles of 94°C for 30 s, 1 min at 59°C, 2 min at 72°C, with a final extension step done at 72°C for 7 min. Sequencing of the target region of *rpoB* in each amplicon was determined by Beckman Coulter Genomics (Danvers, MA) using primer WOG923AP01 (5′-CAG TTC CGC GTT GGC CTG-3′). Utilization of a single pair of oligonucleotide primers for PCR amplification and a single primer for DNA sequencing was possible because 88% of all *rpoB* mutations are localized in the 202 bp region in the middle of the gene [32]. Only base-pair substitutions occurring between positions 1516 and 1717 of the *rpoB* gene were considered

during data analysis. Nucleotide sequences were aligned and analyzed using the ClustalW multiple sequence alignment program (Hinxton, UK). Forward mutations rates in *rpoB* gene (mutations to rifampicin resistance) were determined as previously described [34].

2.7 Western blot analysis of various polA strains

Cells were grown overnight at 37°C in LB plus appropriate antibiotics. The next morning, cultures were diluted 1:100 in fresh LB and grown with aeration at 37°C until they reached an OD_{600} of ~0.5. Cells were harvested by centrifugation, resuspended in 1× SDS sample buffer (50 mM Tris-HCl [pH 6.8], 10% glycerol, 2.3% sodium dodecyl sulfate [SDS], 0.1% bromophenol blue, 10 mM dithiothreitol), and immediately frozen in dry ice. Cells were lysed by multiple freeze-thaw cycles and boiled for 5 min. Extracts were immediately applied to a 4–12% SDS-PAGE gel. After separation proteins were transferred to an Immobilon-P membrane (Millipore) using standard Western blot protocols. The membrane was incubated overnight with a 1:5,000 dilution of affinity purified polyclonal rabbit pol I antisera followed by a 1 hour incubation with a 1:10,000 dilution of secondary goat anti-rabbit alkaline phosphatase conjugated antibodies (BioRad). Steady state levels of pol I was determined using the Tropix CPD-star kit (Applied Biosystems) as previously described [35]

3. Results

3.1 Enzymatic properties of pol I required for ribonucleotide excision repair

The previously characterized biochemical properties of pol I make it an ideal candidate as the enzyme that performs strand displacement synthesis at an RNase HII-generated nick and subsequent removal of the resulting 5′-FLAP. To reconstitute the potential pol I-dependent step of ribonucleotide repair pathway, we synthesized oligonucleotides that after annealing generated a model DNA substrate containing a nick adjacent to a unique uracil ribonucleotide located in double stranded DNA (Fig. 1, substrates I and II) and assayed for the ability of pol I to initiate strand displacement and the subsequent exo/endonuclease removal of the resulting displaced product (Fig. 1).

In this model substrate, the template oligonucleotide contains a single abasic DNA lesion 27 nt downstream of the 3′ template terminus. Pol I bypasses an abasic site poorly and as a consequence, the lesion was intended to provide a kinetic barrier to DNA synthesis and thereby ensure ample time for the 5′ FLAP-endonuclease activity of pol I to cleave the displaced strand. As seen in Fig. 1A, labeling of the 17-mer primer (substrate I) reveals dose-dependent primer extension catalyzed by pol I with the majority of products ultimately terminating opposite the abasic site in the template DNA. At the highest concentration of pol I, in addition to a small amount of full length product indicating replication past the abasic site, accumulation of radiolabeled mono- and short polynucleotides could also be detected. These short products are attributed to the 3′→5′ exonucleolytic proofreading of pol I that is activated when elongation of the radiolabeled primer is interrupted by the lesion in the DNA template.

At most concentrations of pol I, DNA synthesis was generally distributive, with primers only elongated by 1–2 nucleotides. When the 32-mer uracil-containing downstream blocker-strand was radiolabeled (Fig. 1B, substrate II), we observed a dose dependent decrease in the

full-length oligonucleotide with the concomitant accumulation of products that were predominantly 1 nt long, although products of 2–3 nucleotides were also detected. These products form as a result of the efficient 5′-exo/endonuclease activity of pol I removing a single nucleotide, or the short FLAPs generated by limited distributive strand displacement DNA synthesis observed in Fig. 1A. At the highest enzyme to template ratio, when the first two nucleotides of the downstream blocker are displaced relatively efficiently, FLAP-endonuclease activity becomes visible (marked by the appearance of a faint 10 nt long band), but the majority of the released products are still mono-, di-, and trinucleotides.

To mimic substrates with longer 5′-FLAPs (Fig. 1C, substrate III), we synthesized a 27-mer primer oligonucleotide with its 3-end terminating opposite the abasic site in the template. As a′ consequence, the ten 5′ bases of the radiolabelled uracil-containing downstream blocker oligonucleotide cannot hybridize to the template strand and therefore form a FLAP-structure. As seen in Fig. 1C, under these conditions, the 10-nt FLAP substrate was efficiently hydrolyzed by pol I in a concentration-dependent manner. Based upon our observations presented in Fig. 1B, we suggest that pol I-catalyzed excision of errantly incorporated rNMP from DNA mainly proceeds by limited strand displacement synthesis and efficient 5′→3′ exonucleolytic cleavage. However, under conditions where a longer FLAP-structure is generated, the entire DNA FLAP containing a single 5′ ribonucleotide can be removed by the 5′→3′ endonucleolytic activity of pol I.

3.2 Rationale behind the in vivo assays used to elucidate the mechanism of ribonucleotide excision repair

Although pol V is best characterized for its ability to facilitate translesion DNA synthesis, it has also been shown to contribute to high levels of spontaneous mutagenesis on the *E. coli* chromosome in *recA730 lexA*(Def) strains in which the SOS-regulon is fully derepressed and RecA protein is in a constitutively activated form (RecA*) [36]. The mutagenesis is not thought to represent error-prone bypass of cryptic DNA lesions, but rather an ability of the highly activated pol V to compete with *E. coli*'s four other polymerases for access to undamaged genomic DNA [37]. Furthermore, we have shown that such phenotypes can be enhanced when the highly abundant DNA pol IV (encoded by *dinB*) is deleted from the chromosome [5]. Even though our genetic system is quite complex, we believe that its utilization as a tool to investigate RER is justified because expression of the pol V steric gate mutant in *lexA*(Def) *recA730* Δ*dinB* Δ*umuDC* strains maximizes the amount of ribonucleotides errantly incorporated into chromosomal DNA and thus significantly facilitates the task of identifying individual enzymes, as well as cellular pathways involved in protecting genomic DNA from excessive ribonucleotide misincorporation. Indeed, we have successfully used this approach to elucidate the pivotal role of RNase HII in RER [5] and the important back-up roles for RNase HI and NER in ribonucleotide repair [5, 12].

It should be noted that in these studies, we monitored the extent of *umuC*_Y11A-dependent mutagenesis *relative* to that induced by wild-type pol V, rather than the absolute number of His^+ revertants promoted by the mutant polymerase in different backgrounds [5, 12]. The reason for such analysis lies in the fact that the level of mutagenesis promoted by pol V differs considerably in the various strain backgrounds and this variability is likely caused by

the extent of endogenous single-stranded DNA in the cell, which is an important factor in hyper-activating RecA730 and promoting pol V-induced mutagenesis *in vivo* [36, 38]. Since the only known biochemical difference between UmuC_Y11A and wild-type pol V is its ability to incorporate ribonucleotides [2], by expressing *umuC*_Y11A-dependent mutagenesis as a percent of the mutagenesis induced by wild-type pol V, we are able to focus only on those mutagenic events that are specifically related to rNMP incorporation and repair.

In previous studies, we reported that in mismatch- and ribonucleotide-repair proficient *recA730 lexA*(Def) Δ*dinB* strains, *umuC*_Y11A-dependent spontaneous mutagenesis is substantially lower (~10 fold) than that observed with wild-type pol V, but it increases significantly when either MMR, or RER is inactivated [5, 12]. By taking advantage of the fact that MMR complex exhibits a strong bias for the repair of transitions made by all DNA polymerases, while pol V, in contrast to other *E. coli* DNA polymerases, prefers to generate transversions, we demonstrated that the increase in the relative mutagenesis of the strains defective in either MMR, or RER, is determined by unrepaired errors generated by two different polymerases operating in the two different repair pathways [12]. An increase in the relative mutagenesis in the Δ*rnhB* strain represents persistent *umuC*_Y11A-generated mutations escaping RER repair. In contrast, in the MMR-defective strains, it reflects misincorporations made by a DNA polymerase (most probably pol I) catalyzing the re-synthesis step of RNase HII-initiated RER, [12]. As a result, for the current study we have inactivated the MMR pathway via mutations in *mutL*, so as to help identify the *E. coli* DNA polymerase involved in RER.

3.3 Effect of inactivating FLAP-endonuclease activity on ribonucleotide excision repair in vivo

Our biochemical studies demonstrate that pol I possesses all the necessary activities required for RER. One way to prove that pol I is indeed involved in the repair of rNMPs incorporated by plasmid-encoded pol V *in vivo*, is to assay for phenotypic changes in *umuC*_Y11A-dependent mutagenesis in Δ*polA* strains devoid of all pol I's enzymatic activities. Unfortunately, a Δ*polA recA730* strain is inviable [39], thereby precluding the possibility of determining the effects of a Δ*polA* allele on *umuC*_Y11A-dependent mutagenesis. However, certain missense *polA* mutations are viable in a *recA730* strain background. Most relevant, is the *polA107* allele [40] that encodes a Y77C mutant of pol I (Fig. 2A) [41] lacking 5′→3′ exo/endonuclease activity [42], which is viable in the *recA730* background [39]. To assay the role that the 5′→3′ exo/endonuclease of pol I has in ribonucleotide repair, we transduced the *polA107* allele that had previously been linked to *zih219*::Tn*10* into a *recA730 lexA51*(Def) Δ*dinB*Δ *umuDC*Δ *mutL* strain background (Table 1). We reasoned that if the 5′→3′ exo/endonuclease activity is important for pol I's role in ribonucleotide excision repair *in vivo*, then we might observe a difference in the extent of *umuC*_Y11A-dependent spontaneous mutagenesis relative to that promoted by wild-type pol V, in a manner similar to that observed in Δ*rnhB* strains lacking RER [5]. However, that turned out not to be the case, since the relative amount of *umuC*_Y11A-dependent spontaneous mutagenesis remained essentially unchanged in the *polA107* strain (Fig. 2C).

We then considered the possibility that similar to the eukaryotic RER pathway, there may be redundancy at various steps of the *E. coli* RER pathway. Indeed, *E. coli* possesses another FLAP-endonuclease, ExoIX, that is encoded by the *xni* gene [43], and which shares considerable homology to the N-terminus (FLAP-domain) of Pol I [44]. As a consequence, we hypothesized that the FLAP-endonuclease activity of ExoIX might substitute for the FLAP endonuclease activity of pol I during ribonucleotide repair. Although Δ*polA*Δ *xni* strains are apparently inviable [45], we had no difficulty in transducing the Δ*xni725*::Kan allele into either *polA*$^{+}$ or *polA107 recA730 lexA*(Def) Δ*dinB* Δ*umuDC*Δ *mutL* strain backgrounds (Table 1). In both strains, *umuC*_Y11A-dependent mutagenesis was ~25% of that observed with wild-type pol V (Fig. 2C). Thus, inactivation of the 5′-FLAP endonuclease activity of pol I and/or Xni, has no discernible effect of the level of *umuC*_Y11A-dependent mutagenesis *in vivo*. As a consequence, we conclude that in the absence of both 5′→3′ exo/endonuclease of pol I and ExoIX, the single-stranded FLAP generated during RER can be processed by one of the numerous *E. coli* single-stranded nucleases and that the RER does not require a dedicated 5′-FLAP endonuclease.

3.4 Effect of inactivating the 3′→5′ exonuclease activity of pol I on ribonucleotide excision repair in vivo

Since our studies with the *polA107* mutant did not delineate an *in vivo* role for pol I in RER, we assayed the effect of other *polA* missense alleles on *umuC*_Y11A-dependent mutagenesis, and in particular, the D424A mutant that inactivates the 3′→5′ exonuclease activity of pol I (Fig. 2A;[22, 46]. *E. coli* strains with the *polA*_D424A allele expressed from the chromosome exhibit a modest 1.5–4 fold increase in spontaneous mutation frequencies, which have been attributed to error-prone DNA synthesis during the processing of Okazaki fragments [47]. The *polA*_D424A allele was transduced into the *recA730 lexA*(Def) Δ*dinB* Δ*umuDC*Δ *mutL* background and spontaneous His^{+} mutagenesis was assayed. The relative amount of *umuC*_Y11A-specific spontaneous mutagenesis increased from ~22% of that observed in the *polA*$^{+}$ strain, to ~43% in the strain with defective 3′→5′ exonuclease activity (Fig. 2C). We attribute this phenotype to the reduced fidelity of the exonuclease-deficient pol I enzyme making deoxynucleotide misincorporations during RER of *umuC*_Y11A-incorporated ribonucleotides. This observation therefore provides the first clear indication that pol I plays an important role in ribonucleotide excision repair *in vivo*.

3.5 Effect of inactivating the strand-displacement activity of pol I on ribonucleotide excision repair in vivo

In the proposed model for eukaryotic ribonucleotide excision repair, a key step in the process is strand-displacement by pol δ/ε that is initiated at an RNase H2 introduced nick [9]. In *E. coli*, this step is most likely to depend upon the strand-displacement activity of pol I. We therefore hypothesized that a mutant of pol I unable to promote strand-displacement would exhibit a pronounced *umuC*_Y11A phenotype.

Previous *in vitro* studies identified Ser^{769}, Phe^{771}, and Arg^{841} residues in the fingers subdomain of pol I as being required for strand displacement synthesis [27]. Ser^{769} and Phe^{771} are involved in initializing strand separation through the formation of a FLAP-

structured DNA, while Arg^{841} via an interaction with the template strand, ensures optimal strand separation and DNA synthesis [27].

We constructed a *polA* strain defective in strand displacement by introducing S769(AGT) →A769(GCA) and F771(TTC)→A771(GCA) substitutionsinto the chromosomal *polA* gene (Supplemental Fig. 1). Briefly, the chromosomal 3′ *polA* region was first replaced by a Zeo^R cassette, and later substituted by a *polA*_SD^- ::*cat* fragment containing the altered *polA* codons. The correct insertion of the *polA*_SD^- ::*cat* cassette was confirmed by PCR, while the precise replacement of the Zeo^R cassette and introduction of the altered *polA* S769A and F771A codons was confirmed by sequencing of the mutant chromosomal *polA* locus.

The *polA*_SD^- allele was subsequently moved into the *recA730 lexA*(Def) Δ*dinB* Δ*umuDC* Δ*mutL* background by generalized P1 transduction, and spontaneous His^+ mutagenesis assayed. However, the anticipated “pronounced *umuC*_Y11A phenotype” was not observed. Instead, there was essentially no measurable change in the relative amount of *umuC*_Y11A-dependent spontaneous mutagenesis compared to wild-type pol V in the *polA*_SD^- mutant versus the wild-type *polA* strain (Fig. 2C). We attribute the absence of a clear phenotype to the fact that despite exhibiting minimal strand displacement *in vitro* [27], the *polA*_SD^- mutant may nevertheless possess sufficient strand displacement activity to function *in vivo*, or that similar to the 5′→3′ exo/endonuclease activity, which can be assumed by a substitute enzyme, the strand-displacement activity of pol I can be provided by an alternate enzyme, such as another DNA polymerase, and/or one of *E. coli*’s many single-stranded exonucleases.

3.6 Effect of inactivating the DNA polymerase activity of pol I on ribonucleotide excision repair in vivo

During the course of generating the *polA*_SD^- mutant, we made an intermediate construct in which the 3′ end of the *polA* gene was replaced with a Zeocin resistance cassette (*polA*_ΔC; Supplemental Fig. 1). This construct is predicted to express a truncated pol I (residues 1–768) that possesses both 5′→3′ and 3′→5′ exonuclease activities, but should be completely defective in polymerase activity, since it lacks key parts of the “finger” and “palm” domains, which are structural features required for DNA synthesis. Joyce and Grindley previously reported that plasmids encoding either the 5′→3′ and 3′→5′ exonuclease activities of pol I enable *recA*$^+$Δ *polA* strains to grow to form viable colonies on rich LB-media [19]. We therefore attempted to transduce the *polA*_ΔC allele into the *recA730 lexA*(Def) Δ*dinB* Δ*umuDC* background and were able to obtain viable colonies on LB plates.

Western blots with antibodies raised to wild-type pol I indicates that the truncated pol I protein is either poorly expressed, or rapidly degraded, compared to either the wild-type or missense *polA* mutants (Fig. 2B), but nevertheless must be sufficient for cell viability. We assayed spontaneous His^+ mutagenesis and found that the relative amount of *umuC*_Y11A mutagenesis in the *polA*_ΔC strain decreased significantly (p=0.015) when compared to the wild-type *polA* strain (5.3% vs. 22%; Fig. 2C). The decrease in mutagenesis in the *polA*_ΔC polymerase defective strain provides additional evidence for the pivotal role of pol I in RER. We suggest that in the absence of pol I polymerase activity, the DNA synthesis step of RER is performed by an alternate DNA polymerase with higher fidelity than pol I (see below).

3.7 Effect of inactivating rnhB on pol I-dependent ribonucleotide excision repair in vivo

The initial step of RER is Rnase HII-dependent nicking of a DNA duplex 5′ to misincorporated ribonucleotides. In the absence of Rnase HII (as in the case of Δ*rnhB* strains), there is no nicking and subsequently no pol I-dependent RER. As a consequence, *umuC*_Y11A-dependent mutations are therefore expected to persist. To confirm that the change in the extent of mutagenesis observed in the *rnhB*$^+$ 3′→5′ exonuclease-deficient *polA* and *polA*_ΔC strains is due to perturbation of pol I's function in RER, we constructed isogenic Δ*rnhB* strains and assayed for spontaneous His$^+$ mutagenesis.

Since RER is inactive in the Δ*rnhB* strains, *umuC*_Y11A-dependent mutagenesis does not depend on the DNA polymerase performing RER, but rather is determined by persisting mistakes made by the pol V variant. Indeed, the relative extent of *umuC*_Y11A mutagenesis increased from 22% in the *polA*$^+$*rnhB* $^+$strain to 40% in the *polA*$^+$Δ *rnhB* strain. Strikingly, *umuC*_Y11A mutagenesis increased from 5% in the *polA*_ΔC *rnhB*$^+$strain to 48% in the *polA*_ΔC Δ*rnhB* strain (Fig. 3). In contrast, in the *polA*_D424Astrain, activation of RNase HII-dependent RER appears to have little effect on the level of relative mutagenesis (43% vs. 38%). At first glance, this might lead one to conclude that the mutagenesis is independent of RNase HII function. However, this is not the case, but is simply due to the fact that the increase in mutagenesis derived from *polA*_D424A-dependent RER is, by coincidence, in the same range as persisting errors made by *umuC*_Y11A in the absence of RER.

This assumption was confirmed by determining the spectra of spontaneous *rpoB* mutations recovered from the *polA*_D424A *rnhB*$^{+/-}$strains. Indeed, the spectrum of the *polA*_D424A Δ*mutLrnhB* $^+$ strain (Fig. 4A, top; Table 2) is very similar to the spectrum observed by Makiela-Dzbenska *et al* for the Δ*mutL* strain expressing proofreading-deficient pol I [47], while the spectrum of the *polA*_D424A Δ*mutL*Δ *rnhB* strain (Fig. 4A, bottom; Table 2) is reminiscent of the *umuC*_Y11A-dependent spectrum that we previously obtained for the Δ*mutL*Δ *rnhB* strain [12]. An even more dramatic effect of RNase HII on the *rpoB* spectra was observed in the *polA*_ΔC *rnhB*$^{+/-}$ strains completely lacking pol I polymerase activity (Fig. 4B; Table 2). Together, we believe that these observations are consistent with the notion that the spectra of mutations manifested in *rnhB*$^{+/-}$ strains are generated by different DNA polymerases.

Furthermore, we believe that because the extent of mutagenesis observed during RER depends upon the *polA* allele of the strain (increasing ~2-fold in a proofreading-deficient strain and decreasing ~4-fold in a polymerase-deficient strain) it indicates that pol I is the preferred DNA polymerase to facilitate RER in *E. coli*.

3.8 In the absence of pol I, pol III participates in ribonucleotide excision repair in vivo

Only two DNA polymerases (pol II and pol III) remain in the *recA730 lexA*(Def) *polA*_ΔC Δ*dinB* Δ*umuDC* Δ*mutL* strain. To determine which of the two high fidelity polymerases might back up pol I in RER, we constructed a set of isogenic *recA730 lexA*(Def) *polA*_ΔC Δ*dinB* Δ*umuDC* Δ*mutL* strains in which the 3′→5′ exonuclease activity of pol II (*polBex1*), or pol III (*mutD5*), was inactivated and assayed for spontaneous His+ mutagenesis (Fig. 5).

Because these strains lack three of *E. coli*'s five DNA polymerases and exhibit a strong mutator activity caused by defects in mismatch repair and pol II, or pol III-dependent proofreading, they grew poorly and rapidly acquired mutations. As a result, *polBex1* and *mutD5* strains were unable to form colonies on the standard minimal "low histidine" plates normally used for the His^+ reversion assay. This problem was circumvented by substituting the 1μg/ml histidine with 4μg/ml casamino acids, which not only provides the low levels of histidine necessary for the reversion assay, but also all other essential amino acids sufficient for cell viability on the minimal agar plates. Under these assay conditions, one can clearly observe the mutator activity of the *polBex1* and *mutD5* alleles by the dramatic increase in the number of polV-independent His^+ revertants observed in the mutation assays with the strains containing the control vector, pGB2 (Table in Fig. 5).

The data presented in Fig. 5 support the hypothesis that pol I is the polymerase of choice for RER in *E. coli*, since no *umuC*_Y11A mutator effect was observed for either the *polBexI* or *mutD5* exonuclease-deficient alleles in a wild-type $polA^+$ background (relative mutagenesis is ~30% in all these strains). As observed earlier on the low histidine plates (Figs. 2 & 3), the *polA*_ΔC strain exhibited low levels of *umuC*_Y11A mutagenesis on the casamino acids plates (Fig. 5) consistent with the hypothesis that a higher fidelity polymerase assumes the DNA synthesis step of RER in the absence of pol I. Interestingly, mutagenesis increased from just ~2% in the *polA*_ΔC $polB^+$ strain to ~17% in the isogenic *polBex1* strain. Even more striking was an increase in *umuC*_Y11A-dependent mutagenesis in the *polA*_ΔC *mutD5* strain (~63%). We consider such observations as evidence that high-fidelity pol III substitutes for pol I in RER in *polA*_ΔC strains. The statistically significant ($p<0.0001$) increase in mutagenesis observed in the *polBex1* strain may also be interpreted as an indication of a potential back up role for pol II in RER, but is more likely explained by the fact that pol II is an extrinsic proofreader of pol III errors [48].

4. Discussion

pol V is best characterized by its ability to facilitate translesion DNA synthesis. However, in certain genetic backgrounds, it has been shown to contribute to high levels of spontaneous mutagenesis on the undamaged *E. coli* chromosome [36, 37]. We have previously taken advantage of this phenotype, along with a steric gate mutant of pol V that readily incorporates ribonucleotides into the chromosome, to investigate the mechanisms of ribonucleotide repair in *E. coli* [5, 12]. In the present manuscript, we have used the same genetic system to study the potential role of DNA polymerase I in RER. Pol I was chosen as the main focus of the study based upon its previously characterized enzymatic properties that enable it to participate in "nick-translation" [13, 14, 49]. Using model *in vitro* substrates, we demonstrated that pol I can initiate strand displacement synthesis at a nick with a single 5′ terminal ribonucleotide which is removed during repair synthesis by the 5′→3′ exonuclease or 5′-FLAP endonuclease activities of pol I (Figs. 1 & 6).

In *E. coli* strains proficient in RNase H functions, 5′ nicks are generated at sites of errantly incorporated ribonucleotides *in vivo*. The subsequent step in the repair pathway is initiation of DNA synthesis at the nick and strand-displacement of the ribo-containing DNA downstream from the nick (Fig. 6). We provide several compelling lines of evidence that pol

I normally facilitates this step *in vivo*. First, a *polA* mutant deficient in 3′→5′ exonucleolytic activity exhibited significantly (p=0.02) higher levels of *umuC*_Y11A-dependent mutagenesis compared to the isogenic *polA*$^+$ strain (Fig. 2). We theorize that the increased mutagenesis is caused by error-prone pol I-dependent synthesis during RER of *umuC*_Y11A incorporated ribonucleotides (Fig. 5). Secondly, in a *polA*_ΔC strain deficient for pol I DNA synthesis, *umuC*_Y11A-dependent mutagenesis decreased significantly ($p<0.02$; Figs. 2, 3 & 5). We believe that this observation can be explained by the fact that in its absence, another higher fidelity polymerase substitutes for pol I in RER. The observed changes in mutagenesis clearly reflect RER, as they are negated when the pathway is blocked by a Δ*rnhB* allele (Fig. 3) and changes in the spectrum of base substitutions are observed in the *rpoB* gene in the respective strains (Fig. 4).

Our studies with the *mutD5* allele of *dnaQ* and *polBex1* allele of *polB* provide evidence that it is the cell's replicase, pol III, that most likely undertakes the role of pol I in its absence (Fig. 5). Importantly, they also support the model in which pol I is the primary DNA polymerase of choice to facilitate RER, as there is no effect of either the *mutD5* allele or the *polBex1* allele on RER in a *polA*$^+$ strain.

We observed no significant phenotype of a *polA* mutant unable to facilitate strand-displacement, but assume that such activity could readily be performed by pol III, or through the actions of a yet to be determined DNA helicase. Similarly, there was minimal effect of the 5′→3′ exo/endonuclease-deficient *polA107* mutant, even in the absence of another FLAP endonuclease, ExoIX. However, removal of the 5′-FLAP occurs late in the RER process (immediately prior to ligation), and is simply needed to remove regions of single-stranded DNA generated during strand displacement. This step could potentially be facilitated by any number of *E. coli*'s single-stranded exonucleases (Fig. 6).

To elucidate the mechanism of RER in *E. coli*, we have utilized a model system in which a steric gate mutant of pol V with a propensity to incorporate ribonucleotides into DNA has been provided optimal conditions *in vivo*, so as to ensure access to undamaged DNA, where it acts as a molecular "hypodermic needle" to "inject" errant ribonucleotides into the *E. coli* genome. Such an approach has recently allowed us to uncover the mechanisms of ribonucleotide repair that were hitherto unknown ([5, 12] and herein). There is no reason to suspect that the mechanisms of RER uncovered utilizing the *umuC*_Y11A mutant are limited to repair of ribonucleotides specifically incorporated by pol V and given the fact that it has recently been suggested that even high fidelity replicases insert ribonucleotides much more frequently than previously thought [50, 51], we believe that RNase HII-pol I-dependent RER is a global repair pathway that serves as a guardian of the *E. coli* chromosome and protecting it from the potentially harmful consequences of frequent ribonucleotide incorporation during genome duplication.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

pol	DNA polymerase
nt	nucleotide
RER	ribonucleotide excision repair
Top1	topoisomerase 1

References

1. Sale JE, Lehmann AR, Woodgate R. Y-family DNA polymerases and their role in tolerance of cellular DNA damage. Nat Rev Mol Cell Biol. 2012; 13
2. Vaisman A, Kuban W, McDonald JP, Karata K, Yang W, Goodman MF, Woodgate R. Critical amino acids in *Escherichia coli* responsible for sugar discrimination and base-substitution fidelity. Nucleic Acids Res. 2012; 40:6144–6157. [PubMed: 22422840]
3. Brown JA, Suo Z. Unlocking the sugar “steric gate” of DNA polymerases. Biochemistry. 2011; 50:1135–1142. [PubMed: 21226515]
4. Kuban W, Vaisman A, McDonald JP, Karata K, Yang W, Goodman MF, Woodgate R. *Escherichia coli* UmuC active site mutants: effects on translesion DNA synthesis, mutagenesis and cell survival. DNA Repair. 2012; 11:726–732. [PubMed: 22784977]
5. McDonald JP, Vaisman A, Kuban W, Goodman MF, Woodgate R. Mechanisms employed by *Escherichia coli* to prevent ribonucleotide incorporation into genomic DNA by pol V. PLoS Genet. 2012; 8:e1003030. [PubMed: 23144626]
6. Turchi JJ, Huang L, Murante RS, Kim Y, Bambara RA. Enzymatic completion of mammalian lagging-strand DNA replication. Proc Natl Acad Sci USA. 1994; 91:9803–9807. [PubMed: 7524089]
7. Goulian M, Richards SH, Heard CJ, Bigsby BM. Discontinuous DNA synthesis by purified mammalian proteins. J Biol Chem. 1990; 265:18461–18471. [PubMed: 2170412]
8. Rydberg B, Game J. Excision of misincorporated ribonucleotides in DNA by RNase H (type 2) and FEN-1 in cell-free extracts. Proc Natl Acad Sci USA. 2002; 99:16654–16659. [PubMed: 12475934]
9. Sparks JL, Chon H, Cerritelli SM, Kunkel TA, Johansson E, Crouch RJ, Burgers PM. RNase H2-Initiated Ribonucleotide Excision Repair. Mol Cell. 2012; 47:980–986. [PubMed: 22864116]
10. Lazzaro F, Novarina D, Amara F, Watt DL, Stone JE, Costanzo V, Burgers PM, Kunkel TA, Plevani P, Muzi-Falconi M. RNase H and postreplication repair protect cells from ribonucleotides incorporated in DNA. Mol Cell. 2012; 45:99–110. [PubMed: 22244334]
11. Kim N, Huang SN, Williams JS, Li YC, Clark AB, Cho JE, Kunkel TA, Pommier Y, Jinks-Robertson S. Mutagenic processing of ribonucleotides in DNA by yeast topoisomerase I. Science. 2011; 332:1561–1564. [PubMed: 21700875]
12. Vaisman A, McDonald JP, Huston D, Kuban W, Lu L, Van Houten B, Woodgate R. Removal of misincorporated ribonucleotides from prokaryotic genomes: an unexpected role for nucelotide excision repair. PLoS Genet. 2013:e1003878. [PubMed: 24244177]
13. Kornberg, A. DNA Replication. Freeman; New York: 1980.
14. Westergaard O, Brutlag D, Kornberg A. Initiation of deoxyribonucleic acid synthesis. IV. Incorporation of the ribonucleic acid primer into the phage replicative form. J Biol Chem. 1973; 248:1361–1364. [PubMed: 4568814]

15. Derbyshire V, Grindley ND, Joyce CM. The 3′-5′ exonuclease of DNA polymerase I of *Escherichia coli*: contribution of each amino acid at the active site to the reaction. EMBO J. 1991; 10:17–24. [PubMed: 1989882]
16. Polesky AH, Steitz TA, Grindley ND, Joyce CM. Identification of residues critical for the polymerase activity of the Klenow fragment of DNA polymerase I from *Escherichia coli*. J Biol Chem. 1990; 265:14579–14591. [PubMed: 2201688]
17. Lundquist RC, Olivera BM. Transient generation of displaced single-stranded DNA during nick translation. Cell. 1982; 31:53–60. [PubMed: 6218882]
18. Lyamichev V, Brow MA, Dahlberg JE. Structure-specific endonucleolytic cleavage of nucleic acids by eubacterial DNA polymerases. Science. 1993; 260:778–783. [PubMed: 7683443]
19. Joyce CM, Grindley NDF. Method for determining whether a gene of *Escherichia coli* is essential: application to the *polA* gene. J Bacteriol. 1984; 158:636–643. [PubMed: 6233260]
20. Xu Y, Derbyshire V, Ng K, Sun XC, Grindley ND, Joyce CM. Biochemical and mutational studies of the 5′-3′ exonuclease of DNA polymerase I of *Escherichia coli*. J Mol Biol. 1997; 268:284–302. [PubMed: 9159471]
21. Okazaki R, Arisawa M, Sugino A. Slow joining of newly replicated DNA chains in DNA polymerase I-deficient *Escherichia coli* mutants. Proc Natl Acad Sci USA. 1971; 68:2954–2957. [PubMed: 4943548]
22. Makiela-Dzbenska K, Jaszczur M, Banach-Orlowska M, Jonczyk P, Schaaper RM, Fijalkowska IJ. Role of *Escherichia coli* DNA polymerase I in chromosomal DNA replication fidelity. Mol Microbiol. 2009; 74:1114–1127. [PubMed: 19843230]
23. Miller, JH. A short course in bacterial genetics: a laboratory manual and handbook for Escherichia coli and related bacteria. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, N.Y: 1992.
24. Joyce CM, Derbyshire V. Purification of *Escherichia coli* DNA polymerase I and Klenow fragment. Methods Enzymol. 1995; 262:3–13. [PubMed: 8594356]
25. Blattner FR, Plunkett G III, Bloch CA, Perna NT, Burland V, Riley M, Collado-Vides J, Glasner JD, Rode CK, Mayhew GF, Gregor J, Davis NW, Kirkpatrick HA, Goeden MA, Rose DJ, Mau B, Shao Y. The complete genome sequence of *Escherichia coli* K-12. Science. 1997; 277:1453–1462. [PubMed: 9278503]
26. Rutherford K, Parkhill J, Crook J, Horsnell T, Rice P, Rajandream MA, Barrell B. Artemis: sequence visualization and annotation. Bioinformatics. 2000; 16:944–945. [PubMed: 11120685]
27. Singh K, Srivastava A, Patel SS, Modak MJ. Participation of the fingers subdomain of *Escherichia coli* DNA polymerase I in the strand displacement synthesis of DNA. J Biol Chem. 2007; 282:10594–10604. [PubMed: 17259182]
28. Davis BD, Mingioli ES. Mutants of *Escherichia coli* requiring methionine or vitamin B12. J Bacteriol. 1950; 60:17–28. [PubMed: 15436457]
29. Green MHL, Muriel WJ. Mutagen testing using TRP+ reversion in *Escherichia coli*. Mutat Res. 1976; 38:3–32. [PubMed: 768757]
30. Kada T, Brun E, Marcovich H. Comparison of the induction of prototrophic mutants by x-rays and ultraviolet rays in “Escherichia coli” B/r Try. Ann Inst Pasteur (Paris). 1960; 99:547–566. [PubMed: 13791200]
31. Maron DM, Ames BN. Revised methods for the *Salmonella* mutagenicity test. Mutat Res. 1983; 113:173–215. [PubMed: 6341825]
32. Garibyan L, Huang T, Kim M, Wolff E, Nguyen A, Nguyen T, Diep A, Hu K, Iverson A, Yang H, Miller JH. Use of the *rpoB* gene to determine the specificity of base substitution mutations on the *Escherichia coli* chromosome. DNA Repair. 2003; 2:593–608. [PubMed: 12713816]
33. Wolff E, Kim M, Hu K, Yang H, Miller JH. Polymerases leave fingerprints: analysis of the mutational spectrum in *Escherichia coli rpoB* to assess the role of polymerase IV in spontaneous mutation. J Bacteriol. 2004; 186:2900–2905. [PubMed: 15090533]
34. Curti E, McDonald JP, Mead S, Woodgate R. DNA polymerase switching: effects on spontaneous mutagenesis in *Escherichia coli*. Mol Microbiol. 2009; 71:315–331. [PubMed: 19019142]
35. Woodgate R, Ennis DG. Levels of chromosomally encoded Umu proteins and requirements for in vivo UmuD cleavage. Mol Gen Genet. 1991; 229:10–16. [PubMed: 1654503]

36. Sweasy JB, Witkin EM, Sinha N, Roegner-Maniscalco V. RecA protein of *Escherichia coli* has a third essential role in SOS mutator activity. J Bacteriol. 1990; 172:3030–3036. [PubMed: 2188949]
37. Fijalkowska IJ, Dunn RL, Schaaper RM. Genetic requirements and mutational specificity of the *Escherichia coli* SOS mutator activity. J Bacteriol. 1997; 179:7435–7445. [PubMed: 9393709]
38. Vlaši I, Šimatovi A, Kosti KBri. Genetic requirements for high constitutive SOS expression in *recA730* mutants of *Escherichia coli*. J Bacteriol. 2011; 193:4643–4651. [PubMed: 21764927]
39. Fijalkowska I, Jonczyk P, Ciesla Z. Conditional lethality of the *recA441* and *recA730* mutants of *Escherichia coli* deficient in DNA polymerase I. Mutat Res. 1989; 217:117–122. [PubMed: 2645515]
40. Glickman BW, van Sluis CA, Heijneker HL, Rorsch A. A mutant of *Escherichia coli* K12 deficient in the 5′-3′ exonucleolytic activity of DNA polymerase I. I. General characterization. Mol Gen Genet. 1973; 124:69–82. [PubMed: 4354901]
41. Joyce CM, Fujii DM, Laks HS, Hughes CM, Grindley ND. Genetic mapping and DNA sequence analysis of mutations in the *polA* gene of *Escherichia coli*. J Mol Biol. 1985; 186:283–293. [PubMed: 3910840]
42. Heijneker HL, Ellens DJ, Tjeerde RH, Glickman BW, van Dorp B, Pouwels PH. A mutant of *Escherichia coli* K12 deficient in the 5′-3′ exonucleolytic activity of DNA polymerase I. II. Purification and properties of the mutant enzyme. Mol Gen Genet. 1973; 124:83–96. [PubMed: 4582300]
43. Allen LM, Hodskinson MR, Sayers JR. Active site substitutions delineate distinct classes of eubacterial flap endonuclease. Biochem J. 2009; 418:285–292. [PubMed: 19000038]
44. Sayers JR. Computer aided identification of a potential 5′-3′ exonuclease gene encoded by *Escherichia coli*. J Theoret Biol. 1994; 170:415–421. [PubMed: 7996866]
45. Fukushima S, Itaya M, Kato H, Ogasawara N, Yoshikawa H. Reassessment of the *in vivo* functions of DNA polymerase I and RNase H in bacterial cell growth. J Bacteriol. 2007; 189:8575–8583. [PubMed: 17905985]
46. Bebenek K, Joyce CM, Fitzgerald MP, Kunkel TA. The fidelity of DNA synthesis catalyzed by derivatives of *Escherichia coli* DNA polymerase I. J Biol Chem. 1990; 265:13878–13887. [PubMed: 2199444]
47. Makiela-Dzbenska K, Jonczyk P, Schaaper RM, Fijalkowska IJ. Proofreading deficiency of Pol I increases the levels of spontaneous *rpoB* mutations in *E. coli*. Mutat Res. 2011; 712:28–32. [PubMed: 21459099]
48. Banach-Orlowska M, Fijalkowska IJ, Schaaper RM, Jonczyk P. DNA polymerase II as a fidelity factor in chromosomal DNA synthesis in *Escherichia coli*. Mol Microbiol. 2005; 58:61–70. [PubMed: 16164549]
49. Hartman CP, Rabussay D. E. coli DNA polymerase I: enzymatic functions and their application in polymer formation, nick translation and DNA sequencing. Gene Amplif Anal. 1981; 2:17–39. [PubMed: 6765648]
50. Nick McElhinny SA, Watts BE, Kumar D, Watt DL, Lundstrom EB, Burgers PM, Johansson E, Chabes A, Kunkel TA. Abundant ribonucleotide incorporation into DNA by yeast replicative polymerases. Proc Natl Acad Sci USA. 2010; 107:4949–4954. [PubMed: 20194773]
51. Reijns MA, Rabe B, Rigby RE, Mill P, Astell KR, Lettice LA, Boyle S, Leitch A, Keighren M, Kilanowski F, Devenney PS, Sexton D, Grimes G, Holt IJ, Hill RE, Taylor MS, Lawson KA, Dorin JR, Jackson AP. Enzymatic removal of ribonucleotides from DNA is essential for mammalian genome integrity and development. Cell. 2012; 149:1008–1022. [PubMed: 22579044]
52. Watanabe-Akanuma M, Woodgate R, Ohta T. Enhanced generation of A:T->T:A transversions in a *recA730 lexA51*(Def) mutant of *Escherichia coli*. Mutat Res. 1997; 373:61–66. [PubMed: 9015154]
53. Schaaper RM, Cornacchio R. An *Escherichia coli dnaE* mutation with suppressor activity toward mutator *mutD5*. J Bacteriol. 1992; 174:1974–1982. [PubMed: 1548237]
54. Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci USA. 2000; 97:6640–6645. [PubMed: 10829079]

Mutation Research Highlights

We have investigated the mechanism of ribonucleotide excision repair (RER) in *E. coli*.

DNA polymerase I is the primary polymerase of choice for the re-synthesis step of RER.

Back-up enzymes are able to substitute for pol I in the RER pathway *in vivo.*

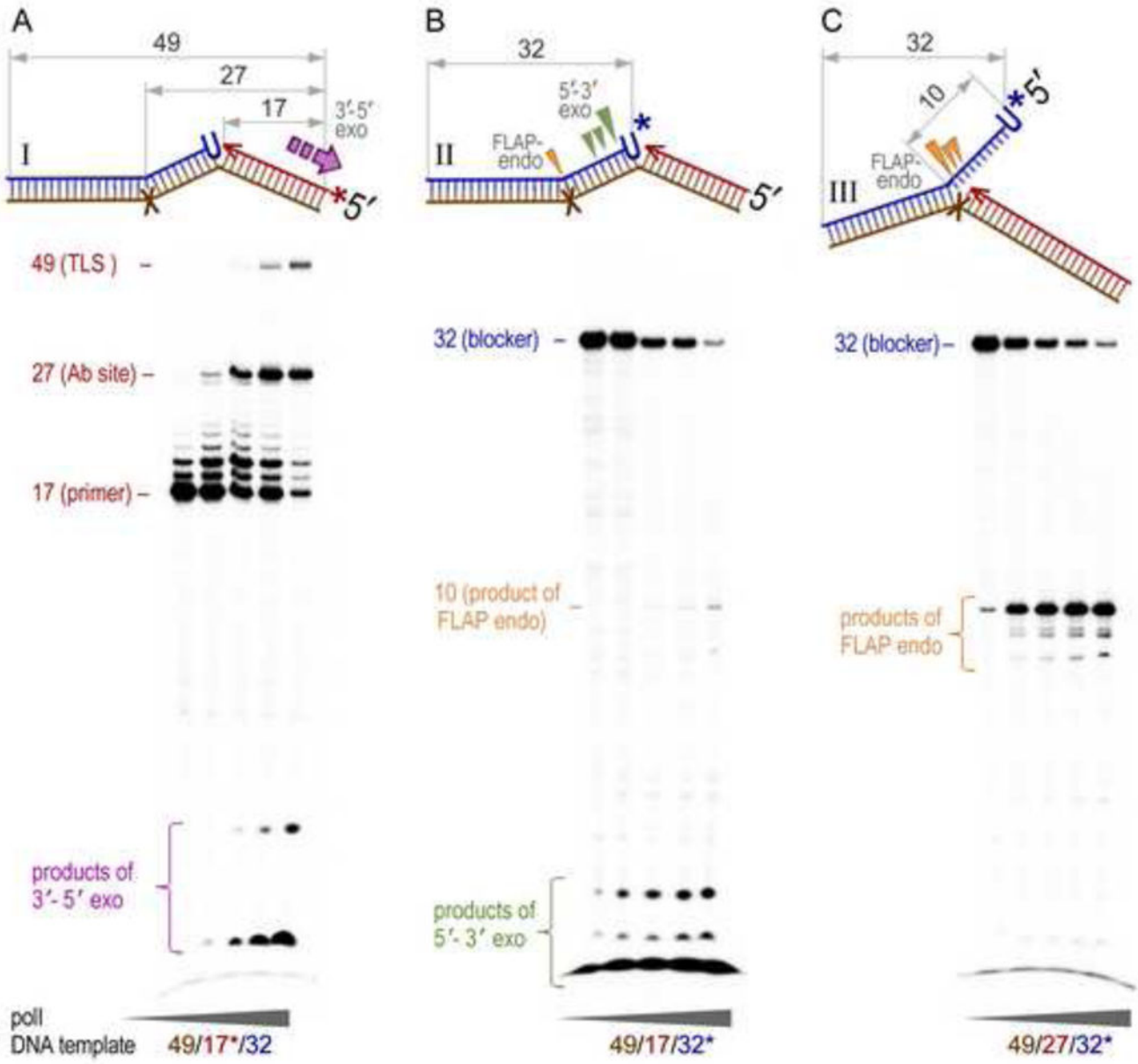
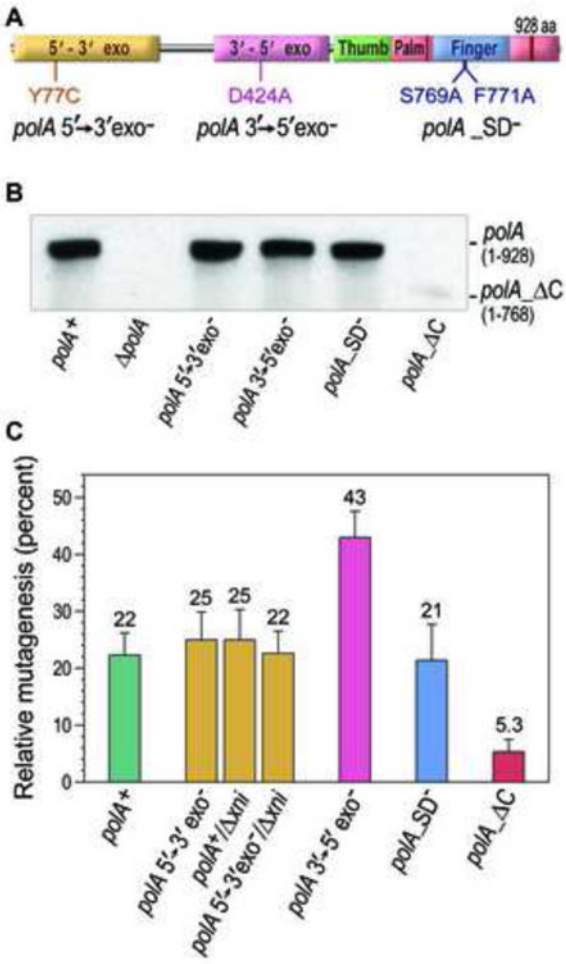


Fig. 1.

Enzymatic activities of pol I on the FLAP endo-, or exonuclease DNA substrates containing a 5′-terminal monoribonucleotide on the downstream blocking oligonucleotide. The pol I-catalyzed strand displacement synthesis and 3′→5′ exonucleolytic proofreading were assayed using nicked DNA substrate generated by annealing of the 49-mer DNA template containing an abasic site (X) with the P^{32}-labeled 17-mer primer and the 32-mer downstream blocker (A). The nick-specific exonuclease (B) and FLAP-specific endonuclease (C) activities of pol I were visualized using the 17- or 27-mer primers, respectively, hybridized together with the P^{32}-labeled 32-mer blocker to the 49-mer DNA template. Structures of DNA substrates are schematically presented on the top of each gel image (brown – template, red – primer, blue - blocker). In substrate I, the 17mer primer is

labeled, while in substrates II and III the 32mer blocking oligonucleotide is labeled (as indicated by, *). The arrows point to the cleavage sites on the DNA made by 3′→5′ exo- (purple ⇪ in I), 5′→3′exo- (green ▼ in II), and flap endonuclease (orange ▼ in II & III) activities of pol I and the corresponding exonucleolytic products, are indicated on the gels. The 27-mer bands seen on the gel in the panel A correspond to the products of primer extension blocked by the abasic site, while the 49-mer band correspond to the primer extensions past the lesion to the full-size product (TLS – translesion synthesis). Reactions were performed using DNA substrate (2 nM), purified pol I (4, 16, 63, 250 pM, or 1 nM), and mixtures of all four dNTPs (100 μM) for 15 min and analyzed by polyacrylamide gel electrophoresis as described in the Materials and Methods section.

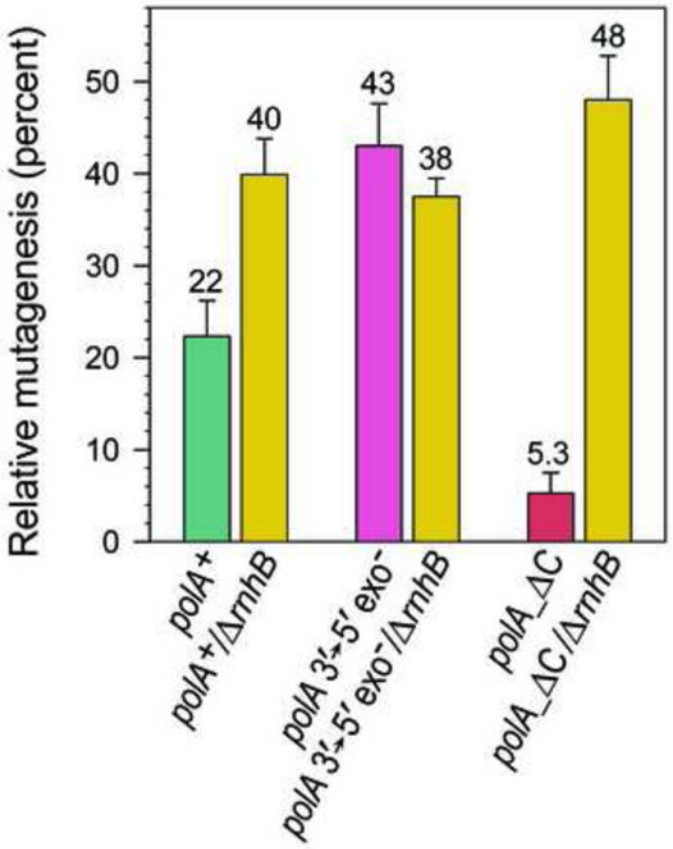


recA730 lexA(Def) Δ*dinB* Δ*umuDC* Δ*mutL*	pGB2	Wt	Y11A
polA⁺	18.8±2.0	1658±173	384±24
polA 5′→3′ exo⁻	28.8±2.1	2131±95	554±15
polA⁺/Δ*xni*	22.8±0.7	1830±54	466±83
polA 5′→3′ exo⁻/Δ*xni*	23.9±1.2	2289±26	536±82
polA 3′→5′ exo⁻	32.3±1.8	1468±100	651±23
*polA*_SD⁻	32.7±5.0	1452±90	305±72
*polA*_ΔC	30.9±4.6	577±44	60±21

Fig. 2.

Effect of mutations in the chromosomal *polA* gene on spontaneous mutagenesis in *recA730 lexA*(Def) Δ*umuDC*Δ *dinB*Δ *mutL* strains harboring the empty vector, pGB2, or pRW134 expressing wild-type pol V, or JM963 expressing UmuD′ and UmuC_Y11A. **A**. The location of the amino acid substitutions of each respective missense *polA* allele are indicated within the structural domains of pol I. The *polA*_ΔC allele expresses a truncated pol I protein (residues 1–768). **B.** Western Blot of steady-state levels of pol I in various strains of *E. coli*. Whole-cell extracts were obtained from the following strains: RW710 (*polA*$^{+}$);

RW900 (Δ*polA*::Kan); (RW1088 (*polA107*); RW1048 (*polA*_D424A); RW1042 (*polA*_F769A/F771A); RW1098 (*polA*_ΔC) and the steady-state levels of pol I determined as described in *Materials and methods*. As observed, the steady state levels of pol I encoded by the three missense *polA* alleles were similar to the level of wild-type pol I. However the steady-state level of the truncated pol I (1–768) protein (encoded by *polA*_ΔC) was significantly lower compared to the wild-type enzyme and missense pol I mutants and was barely detectable unless the images were greatly overexposed (not shown). **C**. Spontaneous mutagenesis was measured by assaying reversion of the *hisG4* ochre allele (leading to histidine prototroph) as described in *Materials and methods.* The following mismatch repair-defective strains were used in the assays: RW710 (*polA*$^+$); RW1088 (*polA107*); RW1050 (Δ*xni*); RW1054 (*polA107*/Δ*xni*); RW1048 (*polA*_D424A); RW1042 (*polA*_F769A/F771A); RW1098 (*polA*_ΔC). The average number of His$^+$ revertants per plate ± standard error of the mean (SEM) for the strains lacking pol V, or expressing wild type, or *umuC*_ Y11A pol V is indicated in the table below the graph. Since the extent of mutagenesis promoted by wild-type pol V differs in the various *polA* strains, the level of mutagenesis promoted by *umuC*_ Y11A is expressed as a percentage of wild-type pol V-dependent mutagenesis in the same strain and shown in the graph. Comparison of these values allows us to characterize the effect of mutations in pol I on the excision repair of ribonucleotides incorporated by the *umuC*_ Y11A allele of pol V. The difference in *umuC*_ Y11A-dependent mutagenesis between *polA*$^+$ and either *polA* 3′→5′ exo$^-$ or *polA*_ΔC strains are statistically significant (p values of 0.02 and 0.015, respectively) as revealed by Student's t-test analysis.



recA730 lexA(Def) Δ*dinB* Δ*umuDC* Δ*mutL*	pGB2	Wt	Y11A
polA^{+}	18.8±2.0	1658±173	384±24
polA^{+}/Δ*rnhB*	22.0±1.0	811±41	322±15
polA 3′→5′ exo^{-}	32.3±1.8	1468±100	651±23
polA 3′→5′ exo^{-}/Δ*rnhB*	19.1±2.6	1002±45	388±3
*polA*_ΔC	30.9±4.6	577±44	60±21
*polA*_ΔC/Δ*rnhB*	8.6±0.8	837±40	408±21

Fig. 3.

Effect of Rnase HII on the level of spontaneous mutagenesis promoted by the empty vector, pGB2, or pRW134 expressing wild-type pol V, or JM963 expressing UmuD′ and UmuC_Y11A in various *polArecA730 lexA* (Def) Δ*umuDC*Δ *dinB*Δ *mutL* ± Δ*rnhB* strains. Spontaneous mutagenesis was measured by assaying reversion of the *hisG4* ochre allele (leading to histidine prototrophy) as described in *Experimental procedures*. The average number of His^{+} revertants per plate ± standard error of the mean (SEM) for the strains lacking pol V, or expressing wild type, or *umuC*_ Y11A pol V is indicated in the table below

the graph. The average level of mutagenesis promoted by *umuC*_ Y11A is expressed as a percentage of wild-type pol V-dependent mutagenesis in the same strain and shown in the graph are taken from [14] The following mismatch repair-defective strains were used in the assays: RW710 (*polA*$^+$); RW1056 (*polA*$^+$Δ *rnhB*); RW1048 (*polA*_D424A); RW1320 (*polA*_D424A Δ*rnhB*); RW1098 (*polA*_ΔC); RW1336 (*polA*_ΔC Δ*rnhB*). The data for the *polA*$^+$, *polA* 3′→5′ exo$^-$ and *polA*_ΔC strains expressing wild-type RNase HII are taken from Fig. 2 and are shown for direct comparison with the data for the Δ*rnhB* strains. The difference in *umuC*_ Y11A-dependent mutagenesis is statistically significant for the *polA*$^+$ and *polA*$^+$/Δ*rnhB* ($p=0.02$) and for *polA*_ΔC and *polA*_ΔC/Δ*rnhB* ($p<0.002$) strains.

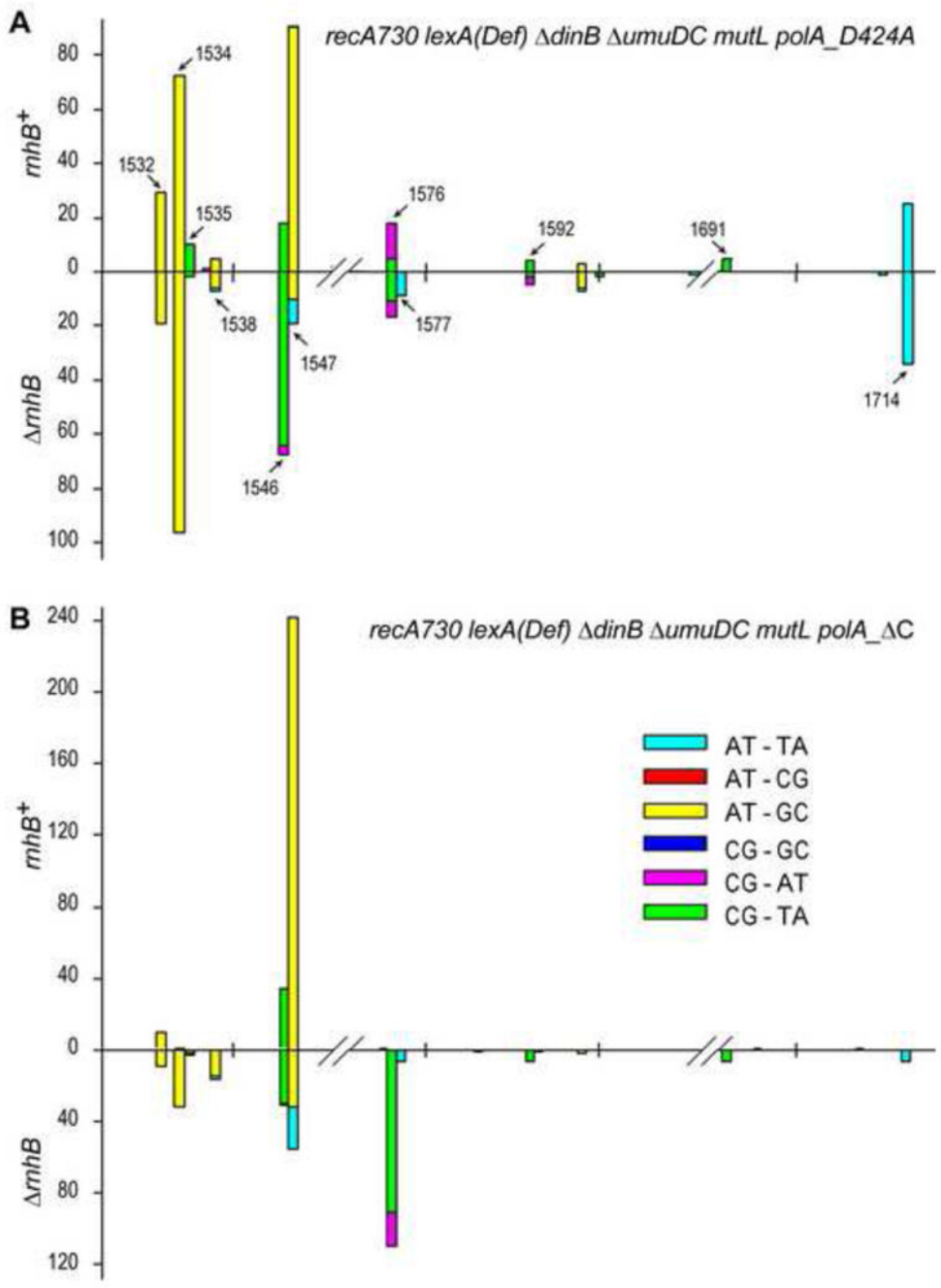
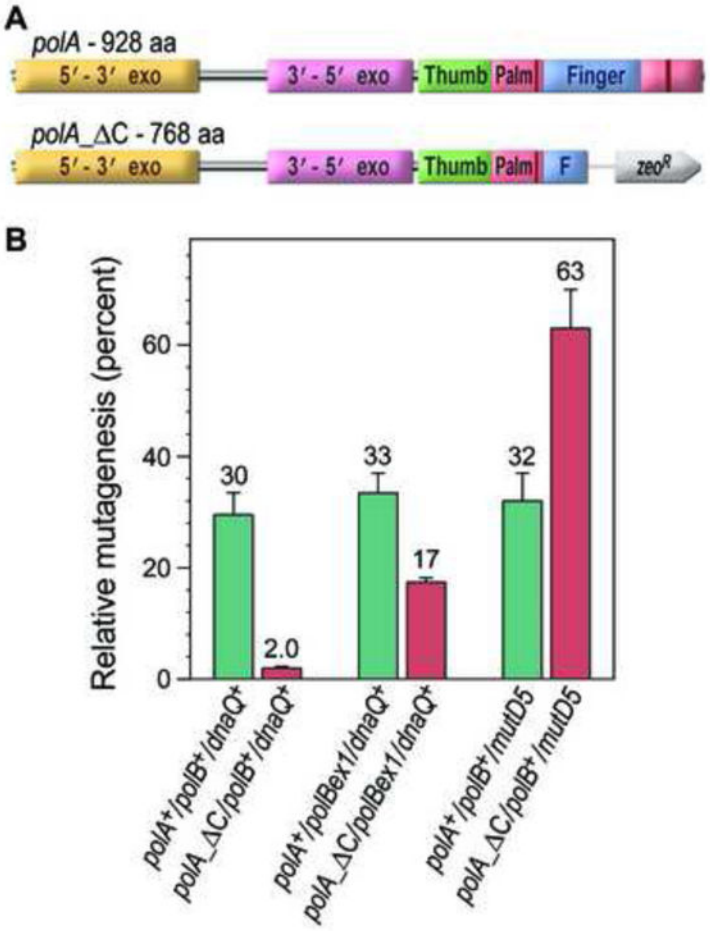


Fig 4.

Spectra of spontaneously arising *rpoB* mutations in *recA730 lexA*(Def) Δ*dinB* Δ*umuDC* Δ*mutLrnhB* +/− strains expressing *umuC*_Y11A. **A.** *polA* _D424A strains deficient in pol I 3′→5′ exonuclease activity. The arrows indicate mutagenic hot spots within the *rpoB*. **B.** *polA* _ΔC strains deficient in pol Ipolymerase activity. Approximately 300 Rif ^{R} mutants were analyzed for each strain (Table 2). The forward mutation rates were assayed by measuring resistance to rifampicin and were in the range of 3–5 × 10^{-6}. The spectra in the *rnhB*+ and Δ*rnhB* strains differ in both the types of mutations and locations of mutagenic

hot-spots. Because of defects in MMR, all the spectra are dominated by transition mutations. However, since low-fidelity pol V makes a significant number of transversion mutations compared to other *E. coli* DNA polymerases [34, 37, 52], the number of *umuC*_Y11A-dependent transversions in the *rnhB* strains is higher than in the *rnhB*$^+$ strains (see Table 2). The shift in the types of mutations observed is significantly more pronounced in the *polA*_ΔC strains. The *rnhB*$^+$ strains undergo active RER; as a consequence, they have a reduced number of pol V-specific transversions, and the spectrum generated in these strains reflect uncorrected errors made by pol I (panel A) or pol III (panel B) during the DNA re-synthesis step of RER.



recA730 lexA(Def) Δ*dinB* Δ*umuDC* Δ*mutL*	pGB2	Wt	Y11A
polA+/*polB*+/*dnaQ*+	27.5±1.9	1407±211	435±5
*polA*_ΔC/*polB*+/*dnaQ*+	28.7±1.4	742±73	43±6
polA+/*polBex1*/*dnaQ*+	148±15	2319±131	871±54
*polA*_ΔC/*polBex1*/*dnaQ*+	462±32	725±76	507±38
polA+/*polB*+/*mutD5*	26.3±1.9	1819±156	608±52
*polA*_ΔC/*polB*+/*mutD5*	231±60	386±75	328±18

Fig. 5.

Effect of *polBex1* and *mutD5* alleles on spontaneous mutagenesis in *recA730 lexA*(Def) Δ*umuDC*Δ *dinB*Δ *mutLpolA* $^{+}$/ *polA*_ΔC strains harboring the empty vector, pGB2, or pRW134 expressing wild-type pol V, or JM963 expressing UmuD′ and UmuC_Y11A. **A**. Full-length *polA* encodes a 928 amino acid protein, whereas the *polA*_ΔC allele expresses a truncated pol I protein (residues 1–768) that lacks key structural domains necessary to catalyze DNA synthesis. **B**. Because of their low viability on the defined “low histidine” minimal plates, spontaneous mutagenesis was measured by assaying reversion of the *hisG4*

ochre allele (leading to histidine prototrophy) on minimal agar plates containing 4μg/ml Casamino acids as described in *Materials and methods*. The following mismatch repair-defective strains were used in the assays: RW1236 (*polA*$^+$); RW1098 (*polA*_ΔC); RW1226 (*polA*$^+$*polBex1*); RW1324 (*polA*_ΔC *polBex1*); RW1250 (*polA*$^+$*mutD5*); RW1218 (*polA*_ΔC *mutD5*). The average number of His$^+$ revertants per plate ± standard error of the mean (SEM) for the strains lacking pol V, or expressing wild type, or *umuC*_ Y11A pol V is indicated in the table below the graph. Since the extent of mutagenesis promoted by wild-type pol V differs in the isogenic strains, the level of mutagenesis promoted by *umuC*_ Y11A is expressed as a percentage of wild-type pol V-dependent mutagenesis in the same strain and shown in the graph. Comparison of these values allows us to characterize the effect of inactivating the proofreading activity of pol II (*polBex1*), or proofreading-deficient pol III (*mutD5*), on the excision repair of ribonucleotides incorporated by the *umuC*_ Y11A allele of pol V in *polA*$^+$ or *polA*_ΔC strains.

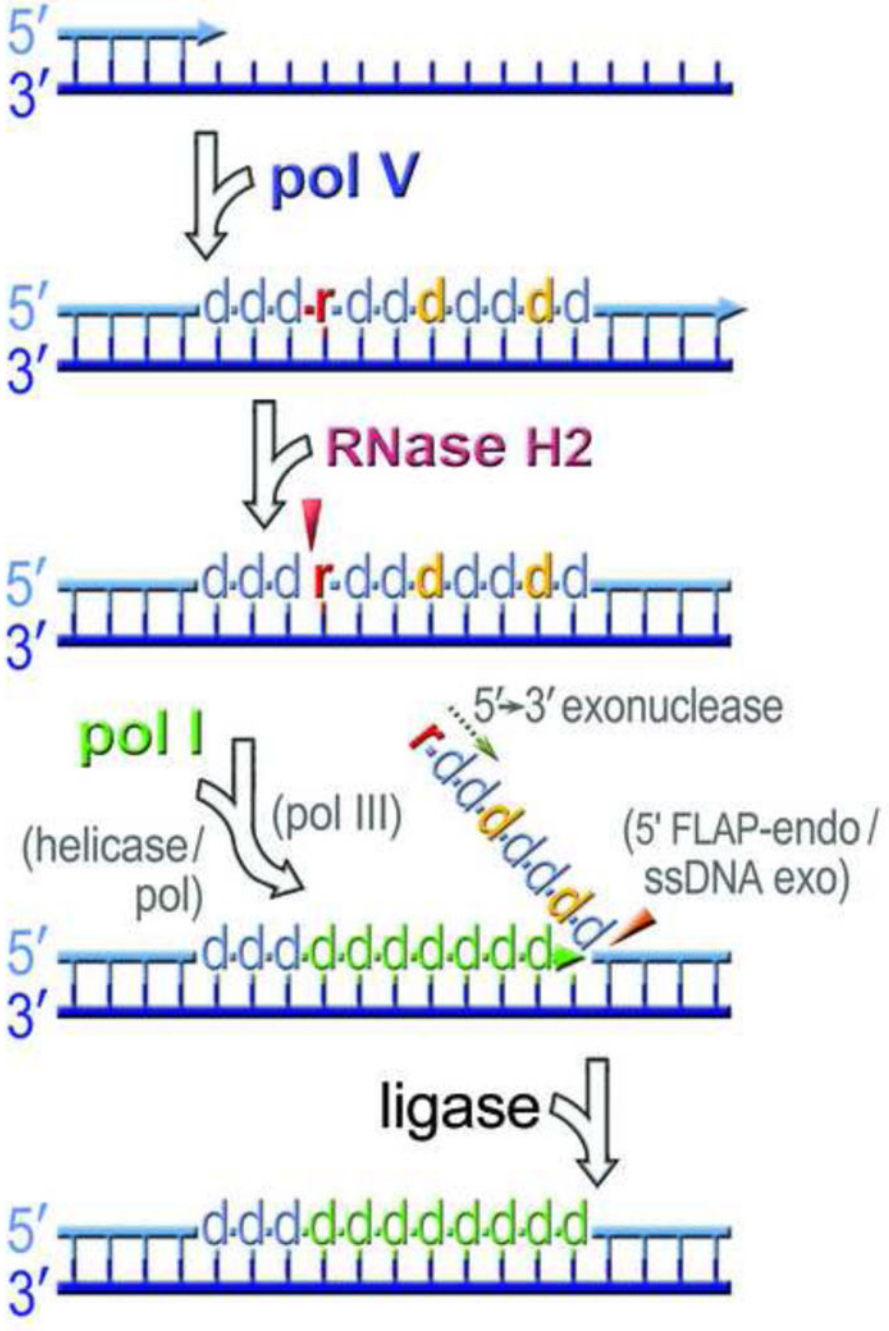


Fig. 6.

A model for ribonucleotide excision repair in *E. coli*. Pol V introduces multiple base substitutions (shown as *d* in orange) when it gains access to undamaged chromosomal DNA. It can also incorporate ribonucleotides (shown as *r* in red) into the nascent DNA strand. RNase HII incises the bond at the rNMP/dNMP junction 5′ to the ribonucleotide, generating DNA containing a single stranded break with 5′-phospho-ribonucleotide and 3′-hydroxyl ends. Pol I commences DNA synthesis at the nick and promotes strand displacement of the mutagenic pol V tract (shown as *d* in green). In *polA* mutants that are unable to promote

strand displacement, the unwinding of the nicked intermediate proceeds through the action of an unknown DNA helicase, or polymerase (shown in parentheses). In *polA*_ΔC strains deficient for DNA synthesis, the 3′-hydroxyl end of the nicked intermediate is extended by high-fidelity pol III (shown in parentheses). The displaced region containing rNMP and misincorporated dNMPs is usually degraded by the 5′→3′ exonuclease or 5′-FLAP endonuclease activity of pol I, but in its absence, the displaced strand may be degraded by the FLAP endonuclease Xni, or another (yet to be identified) single-stranded nuclease (shown in parentheses). Complete repair occurs once the nick is sealed by DNA ligase. Although this model is based upon our present studies with the error-prone *umuC*_Y11A steric gate mutant of pol V, we envisage that identical processes occur when high-fidelity DNA polymerases inadvertently incorporate ribonucleotides into genomic DNA.

Table 1

E. coli strains used in this study

Strain	Relevant Genotype	Source or Reference
MG1655	F- λ^- *rph-*	*E. coli* Genetic Stock Center
MG1655 *polA*::Zeo	F- λ^- *rph- polA*_ΔC [b]	This work
MG1655 *polA*_SD$^-$	F- λ^- *rph polA_F769A/F771A ::cat*	This work
KA797	*polA_D424A ::cat*	[22]
RW900[a]	Δ*polA*::Kan	LGI stocks
KM52	Δ*mutL460::cat*	Martin Marinus
JW4128	Δ*mutL720*::Kan	*E. coli* Genetic Stock Center
ES1484	*mutL218*::Tn*10*	*E. coli* Genetic Stock Center
JW0178	Δ*rnhB782*::Kan	*E. coli* Genetic Stock Center
JW5446	Δ*xni725*::Kan	*E. coli* Genetic Stock Center
JW0221	Δ*dinB749*::Kan	*E. coli* Genetic Stock Center
NR9548	*mutD5 zaf13*::Tn*10*	[53]
EMBL1789	*polA107*	*E. coli* Genetic Stock Center
ET1214	*zih-219*::Tn*10*	*E. coli* Genetic Stock Center
RW896	*polA107 zih-219*::Tn*10*	EMBL1789 x P1. ET1214
RW584[a]	*recA730 lexA51*(Def) Δ*(umuDC)596::ermGT*	[5]
RW698[a]	*recA730 lexA51*(Def) Δ*(umuDC)596::ermGT* Δ*dinB61::ble*	[5]
RW710[a]	*recA730 lexA51*(Def) Δ*(umuDC)596::ermGT* Δ*dinB61::ble mutL215*::Tn*5*	[34]
RW928[a]	*recA730 lexA51*(Def) Δ*(umuDC)596::ermGT* Δ*dinB61::ble polA_D424A ::cat*	RW698 x P1.KA797
RW1048[a]	*recA730 lexA51*(Def) Δ*(umuDC)596::ermGT* Δ*dinB61::ble polA_D424A ::cat* Δ*mutL720*::Kan	RW928 x P1.JW4128
RW932[a]	*recA730 lexA51*(Def) Δ*(umuDC)596::ermGT* Δ*dinB61::ble* Δ*xni725*::Kan	RW698 x P1 JW5446
RW1050[a]	*recA730 lexA51*(Def) Δ*(umuDC)596::ermGT* Δ*dinB61::ble* Δ*xni725*[c] Δ*mutL720*::Kan	RW932 x P1.JW4128
RW948[a]	*recA730 lexA51*(Def) Δ*(umuDC)596::ermGT* Δ*dinB61::ble polA107 zih-219*::Tn*10*	RW698 x P1. RW896
RW1088[a]	*recA730 lexA51*(Def) Δ*(umuDC)596::ermGT* Δ*dinB61::ble polA107 zih-219*::Tn*10* Δ*mutL720*::Kan	RW948 x P1. JW4128
RW968[a]	*recA730 lexA51*(Def) Δ*(umuDC)596::ermGT* Δ*dinB61::ble polA107 zih-219*::Tn*10* Δ*xni725*::Kan	RW948 x P1. JW5446
RW1054[a]	*recA730 lexA51*(Def) Δ*(umuDC)596::ermGT* Δ*dinB61::ble polA107 zih-219*::Tn*10* Δ*xni725*::Kan Δ*mutL460::cat*	RW968 x P1. KM52
RW1030[a]	*recA730 lexA51*(Def) Δ*(umuDC)596::ermGT* Δ*dinB61::ble polA_F769A/F771A ::cat*	RW698 x P1. MG1655*polA*_SD$^-$
RW1042[a]	*recA730 lexA51*(Def) Δ*(umuDC)596::ermGT* Δ*dinB61::ble polA_F769A/F771A ::cat* Δ*mutL720*::Kan	RW1030 x P1. JW4128
RW1060[a]	*recA730 lexA51*(Def) Δ*(umuDC)596::ermGT polA*_ΔC [b]	RW584 x P1. MG1655*polA*::Zeo
RW1086[a]	*recA730 lexA51*(Def) Δ*(umuDC)596::ermGT* Δ*dinB749*::Kan *polA*_ΔC [b]	RW1060 x P1. JW0221
RW1098[a]	*recA730 lexA51*(Def) Δ*(umuDC)596::ermGT* Δ*dinB749*::Kan *polA*_ΔC [b] Δ*mutL460::cat*	RW1086 x P1. KM52
RW838[a]	*recA730 lexA51*(Def) Δ*(umuDC)596::ermGT* Δ*dinB61::ble* Δ*rnhB782*::Kan	[5]
RW970[a]	*recA730 lexA51*(Def) Δ*(umuDC)596::ermGT* Δ*rnhB782* Δ*dinB61::ble*	Kan^S derivative of RW838[c]

Strain	Relevant Genotype	Source or Reference
RW1056[a]	*recA730 lexA51*(Def) Δ(*umuDC*)*596::ermGT* Δ*rnhB782* Δ*dinB61::ble* Δ*mutL720*::Kan	RW970 x P1 JW4128
RW1318[a]	*recA730 lexA51*(Def) Δ(*umuDC*)*596::ermGT* Δ*rnhB782* Δ*dinB749*::Kan	RW970 x P1. JW0221
RW1326[a]	*recA730 lexA51*(Def) Δ(*umuDC*)*596::ermGT* Δ*rnhB782* Δ*dinB749*::Kan *polA*_ΔC [b]	RW1318 x MG1655*polA*::Zeo
RW1336[a]	*recA730 lexA51*(Def) Δ(*umuDC*)*596::ermGT* Δ*dinB749*::Kan Δ*rnhB782 polA*_ΔC [b] Δ*mutL460::cat*	RW1326 x KM52
RW1214[a]	*recA730 lexA51*(Def) *polBex1* Δ(*umuDC*)*596::ermGT* Δ*dinB749*::Kan	LGI lab stocks
RW980[a]	*recA730 lexA51*(Def) Δ(*umuDC*)*596::ermGT* Δ*dinB61::ble polA_D424A ::cat* Δ*rnhB782*::Kan	RW928 x P1. JW0178
RW1320	*recA730 lexA51*(Def) Δ(*umuDC*)*596::ermGT* Δ*dinB61::ble polA_D424A ::cat* Δ*rnhB782*::Kan *mutL218*::Tn*10*	RW980 x P1.ES1418
RW1214[a]	*recA730 lexA51*(Def) *polBex1* Δ(*umuDC*)*596::ermGT* Δ*dinB749*::Kan	LGI stocks
RW1224[a]	*recA730 lexA51*(Def) *polBex1* Δ(*umuDC*)*596::ermGT* Δ*dinB749*	Kan^S derivative of RW1214[c]
RW1226[a]	*recA730 lexA51*(Def) *polBex1* Δ(*umuDC*)*596::ermGT* Δ*dinB749* Δ*mutL720*::Kan	RW1224 x P1.JW4128
RW1242[a]	*recA730 lexA51*(Def) *polBex1* Δ(*umuDC*)*596::ermGT* Δ*dinB749 polA*_ΔC [b]	RW1224 x MG1655*polA*::Zeo
RW1324[a]	*recA730 lexA51*(Def) *polBex1* Δ(*umuDC*)*596::ermGT* Δ*dinB749 polA*_ΔC [b] Δ*mutL460::cat*	RW1242 x KM52
RW1218[a]	*recA730 lexA51*(Def) Δ(*umuDC*)*596::ermGT* Δ*dinB749*::Kan *polA*_ΔC [b] Δ*mutL460::cat mutD5 zaf13*::Tn*10*	RW1098 x P1. NR9548
RW1250[a]	*recA730 lexA51*(Def) Δ(*umuDC*)*596::ermGT* Δ*dinB61::ble* Δ*mutL720*::Kan *mutD5 zaf13*::Tn*10*	RW1236 x P1. NR9548

[a]: thr-1 araD139 Δ(gpt-proA)62 lacY1 tsx-33 glnV44 galK2 hisG4 rpsL31 xyl-5 mtl-1 argE3 thi-1 sulA211

[b]: encodes *polA* residues 1–768 and expresses 5′→3′ exo/endonuclease and 3′→5′ exonuclease functions of pol I.

[c]: The Kan^R cassette was “Flp’d” out by transforming the strain with the temperature sensitive plasmid, pCP20 encoding the FLP recombinase, followed by overnight incubation at 43°C [54].

Table 2

Spectrum of spontaneous mutations generated in the *rpoB* gene of *recA730 lexA*(Def) Δ*dinB*Δ *umuDC*Δ *mutLrnhB* $^{+}$/Δ*rnhB polA*_D424A and *polA*_ΔC strains in the presence of *umuC*_Y11A[a].

	Position [b]	bp change	*rnhB*$^{+}$ *polA*_D424A	Δ*rnhB* *polA*_D424A	*rnhB*$^{+}$ *polA*_ΔC	Δ*rnhB* *polA*_ΔC
	1532	AT→GC	29	19	10	9
	1534	AT→GC	72	96	1	32
[	1535	CG→AT				1
[	1535	CG→TA	10	2		2
	1537	CG→AT	1			
[	1538	AT→TA		1		1
[	1538	AT→GC	5	6		15
[	1546	CG→AT		3		1
[	1546	CG→TA	18	64	34	30
[	1547	AT→TA	1	9		23
[	1547	AT→GC	90	10	242	32
	1552	AT→GC	2	2	1	1
	1565	CG→TA				1
	1575	CG→GC			1	
[	1576	CG→AT	13	6		19
[	1576	CG→TA	5	11		91
	1577	AT→TA		9		6
	1586	CG→TA				1
[	1592	CG→AT		3		
[	1592	CG→TA	4	2		6
	1593	CG→TA				1

	Position [b]	bp change	*rnhB*$^+$ *polA*_D424A	Δ*rnhB* *polA*_D424A	*rnhB*$^+$ *polA*_ΔC	Δ*rnhB* *polA*_ΔC
[	1598	AT→TA		1		
	1598	AT→GC	3	6		2
	1600	CG→TA		2		
	1611	CG→TA		1		
	1691	CG→TA	5			6
	1695	AT→GC			1	
	1708	CG→TA			1	
	1711	CG→TA		1		
	1714	AT→TA	25	34		6
		Transitions	243 (86%)	221 (77%)	290 (99%)	229 (80%)
		Transversions	40 (14%)	67 (23%)	1 (0.3%)	57 (20%)
		Total	283	288	291	286

[a]: The data are the number of mutants found for each type of base substitution at a particular position

[b]: The numbering system originates from Garibyan et al., [32], where the A of the ATG initiation codon is #1.