DNA polymerase switching: effects on spontaneous mutagenesis in Escherichia coli

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Summary

Escherichia coli possesses five known DNA polymerases (pols). Pol III holoenzyme is the cell's main replicase, while pol I is responsible for the maturation of Okazaki fragments and filling gaps generated during nucleotide excision repair. Pols II, IV and V are significantly upregulated as part of the cell's global SOS response to DNA damage and under these conditions, may alter the fidelity of DNA replication by potentially interfering with the ability of pols I and III to complete their cellular functions. To test this hypothesis, we determined the spectrum of rpoB mutations arising in an isogenic set of mutL strains differentially expressing the chromosomally encoded pols. Interestingly, mutagenic hot spots in rpoB were identified that are susceptible to the actions of pols I–V. For example, in a recA730 lexA(Def) mutL background most transversions were dependent upon pols IV and V. In contrast, transitions were largely independent upon pol I and to a lesser extent, pol III. Furthermore, the extent of pol I-dependent mutagenesis at one particular site was modulated by pols II and IV. Our observations suggest that there is considerable interplay among all five E. coli polymerases that either reduces or enhances the mutagenic load on the E. coli chromosome.

Introduction

Cellular survival relies upon the ability of an organism to faithfully duplicate its genome. While an occasional mutation may prove to be a selective advantage in evolutionary terms, most mutations are deleterious. Indeed, replication errors in humans often lead to genetically inheritable diseases, the onset of cancer and premature ageing (Kunkel, 2003; McCulloch and Kunkel, 2008). As a consequence, how a cell chooses a particular DNA polymerase (pol) to maintain its genomic integrity is of great interest. In humans, it is particularly challenging, as the genome is now believed to encode at least 15 DNA polymerases (Bebenek and Kunkel, 2002). As might be expected, lower life forms tend to possess fewer polymerases, but as in the case of Escherichia coli, there are still five to choose from, which is nevertheless a daunting task.

Escherichia coli pol I was the first DNA polymerase ever identified (Bessman et al., 1956). It is encoded by the polA gene (De Lucia and Cairns, 1969) and is estimated to be present at an intracellular concentration of ~400 molecules per cell (Komberg and Baker, 1992). The single 103 kDa pol I polypeptide not only exhibits polymerase activity, but 5'→3' and 3'→5' exonuclease activities (Komberg and Baker, 1992). Pol I's primary role is to remove RNA primers and fill Okazaki fragments generated during lagging strand DNA synthesis (Okazaki et al., 1971), but it also fills gaps generated during nucleotide excision repair of damaged DNA (Cooper and Hanawalt, 1972; Komberg and Baker, 1992; Friedberg et al., 2006). ΔpolA strains are inviable when grown in rich medium, but are viable if the 5'→3' or 3'→5' exonuclease functions are provided in trans (Joyce and Grindley, 1984), indicating that exonuclease and not polymerase functions are primarily required for viability.

Pol II activity was first described in 1971 (Komberg and Gefter, 1971; Gefter et al., 1971), but it was not until 1990 that the polB gene encoding pol II was shown to be allelic with the LexA-regulated and damage-inducible dinA gene (Bonner et al., 1990; Iwasaki et al., 1990). In an uninduced cell, pol II is expressed at a basal level of ~50 molecules per cell (Qiu and Goodman, 1997) and these levels increase approximately sevenfold upon DNA damage (Bonner et al., 1988). The single 88 kDa pol II polypeptide also exhibits for both polymerase and 3'→5' exonuclease activities (Cai et al., 1995) and as a consequence, pol II-dependent replication is reasonably accurate with misincorporations occurring in the 1 × 10^-6 range (Cai et al., 1995). For many years, the cellular role of pol...
II remained enigmatic, but it is now known that the polymerase plays an important role in replication restart (Rangarajan et al., 1999; 2002) and translesion replication (Napolitano et al., 2000; Becherel and Fuchs, 2001). It has also been suggested that pol II’s proofreading activity may also help maintain E. coli’s genomic integrity by removing replication errors generated by pol III (Banach-Orlowska et al., 2005).

Pol III holoenzyme is the cell’s main replicase (Getler et al., 1971; Wechsler et al., 1973; McHenry and Kornberg, 1977; Kornberg and Baker, 1992). The holoenzyme is encoded by 10 separate genes, which combine to form a 15-polypeptide replicase (McHenry, 1991; 2003; O’Donnell, 2006; Pomerantz and O’Donnell, 2007). Pol III holoenzyme is highly processive and the estimated 10–20 molecules per cell are sufficient to facilitate duplication of the entire ~4 Mbp E. coli genome. The 130 kDa α-catalytic subunit is encoded by dnaE (Getler et al., 1971) and temperature-sensitive missense mutations in dnaE often exhibit elevated spontaneous mutation rates, even at permissive temperature (Hall and Brammar, 1973; Wechsler et al., 1973; Sevastopoulos and Glaser, 1977; Konrad, 1978).

Pol IV activity was first reported in 1999 and shown to be encoded by the damage-inducible dinB gene (Wagner et al., 1999). However, the LexA-binding site within the dinB operator varies considerably from the consensus binding site (Fernandez de Henestrosa et al., 2000). As a consequence, pol IV is expressed at high basal levels, with an estimated intracellular concentration of ~250 molecules per undamaged cell and these levels increase further a further 10-fold upon DNA damage (Kim et al., 2001). The enzyme lacks intrinsic 3′→5′ exonuclease activity and is a low-fidelity enzyme with a misincorporation frequency in the 10^{-3}–10^{-4} range (Tang et al., 2000). While it is believed that basal levels of error-prone pol IV do not contribute significantly to spontaneous mutagenesis in dividing cells (Wolff et al., 2004; Kuban et al., 2005; Tago et al., 2005), stationary phase cells exhibit dinB-pol IV-dependent mutagenesis (McKenzie et al., 2001; Tompkins et al., 2003) and overproduction of pol IV increases ~1 frame-shift mutagenesis in exponentially growing cells (Kim et al., 1997; Wagner and Nohmi, 2000; Kuban et al., 2005). In addition, it has been recently suggested that pol IV may also contribute to SOS-dependent spontaneous base-pair substitution mutagenesis (Kuban et al., 2006).

Pol V was the last E. coli polymerase identified (Tang et al., 1999). The enzyme is encoded by umuDC (Tang et al., 1999), and consists of a dimer of the post-translationally modified UmuD’ protein in a complex with UmuC (Woodgate et al., 1989; Reuven et al., 1999; Tang et al., 1999). Like dinA/polIB and dinB/pol IV the umuDC genes are upregulated in response to DNA damage (Bagg et al., 1981). However, unlike pol II and pol IV, pol V is expressed at very low basal levels. Indeed, LexA-mediated transcriptional control, combined with RecA-mediated intermolecular auto-cleavage of UmuD (McDonald et al., 1998) required to activate UmuD’ (Nohmi et al., 1988), as well as Lon- and CipXP-mediated proteolysis of the Umu proteins (Frank et al., 1996; Gonzalez et al., 1998; 2000), limits the number of functionally active pol V molecules in an undamaged cell to 15 or less (Woodgate and Ennis, 1991). Levels of pol V do, however, increase upon DNA damage, but at most reach a maximum of 200 molecules per wild-type cell, which is roughly equivalent to basal levels of pol IV expression (Woodgate and Ennis, 1991; Kim et al., 2001). Like pol IV, pol V lacks intrinsic 3′→5′ proofreading activity and is also considered a low-fidelity polymerase (Tang et al., 2000). Despite its low cellular concentration, pol V is responsible for most damage-induced mutagenesis in E. coli as strains carrying certain missense mutations in umuD or umuC (Kato and Shinoura, 1977; Steinborn, 1978) or a deletion of the entire umuDC operon (Woodgate, 1992) are essentially rendered non-mutable, even after exposure to a wide variety of known mutagens/carcinogens.

All five E. coli pols bind the replicative β-clamp and this interaction is required for both processive synthesis in vitro and the respective enzyme’s cellular functions (Bonner et al., 1992; Dalrymple et al., 2001; Lopez de Saro and O’Donnell, 2001; Pham et al., 2001; Wagner et al., 2001; Becherel et al., 2002; Lenne-Samuel et al., 2002; Bunting et al., 2003). While pol I (polA) and pol III (dnaE) are essential, strains carrying deletions of pol II (polB), pol IV (dinB) or pol V (umuDC) are viable, but strains lacking one or more of the pols are unable to compete with polymerase-proficient bacteria during the stationary phase of the bacterial life cycle, indicating that the three damage-inducible polymerases each contribute to the overall selective fitness of E. coli (Yeiser et al., 2002).

Given its high fidelity and processivity, it is generally assumed that pol III facilitates duplication of the E. coli chromosome with the assistance of pol I to fill Okazaki fragments (Kornberg and Baker, 1992). However, recent studies with replication-impaired pol III mutants suggest that pols II, IV and V may have considerable access to the E. coli chromosome. To do so, we analysed the spectrum of missense mutations generated in polB in an isogenic set of mutl strains differentially expressing one or more of E. coli’s five pols. Our study was facilitated...
by the fact that each polymerase leaves a unique genetic ‘fingerprint’ (Wolff et al., 2004) and that the strains utilized lack methyl-directed mismatch repair, so that we could follow polymerase-specific misincorporation events, rather than assay those events simply remaining after repair (Schaaper and Dunn, 1987). Interestingly, in the various polymerase-deficient backgrounds assayed, the location and magnitude of the base substitution hot spots changed, suggesting that under certain conditions, there is considerable interplay between E. coli’s pols that ultimately either reduces or enhances the mutagenic load on the E. coli genome.

### Results

#### Mutation rates in the various MutL strains

The strains used in this study harbour Tn5 or Tn10 insertions in mutL, and are deficient in postreplicative methyl-directed mismatch repair (Table 1). As a consequence, they exhibit mutation rates that are ~100–200-fold higher than a mismatch-proficient strain (Table 2). In general, the mutation rates of the various mutL strains were similar and only varied from the parental recA+/lexA+/mutL strain, RW720, by a factor of two- to fourfold (Table 2). The spectra of rpoB mutations were obtained from ~350–400 mutL mutants arising in each strain background.

#### Spectra of rpoB mutations in the presence of differential levels of E. coli’s five pols

The spectrum of spontaneous rpoB base-pair substitutions observed in a recA− lexA+/mutL background is shown in Fig. 1A. As expected, transitions accounted for more than 90% of the total mutations observed (Table 3). Base-pair substitutions were located at 10 sites, with four prominent hot spots (defined as sites at which there are 10 or more mutations). These were AT→GC transitions

### Table 1. E. coli strains used in this work.

| Strain designation | Relevant genotype/phenotype |
|--------------------|-----------------------------|
| RW118              | recA− lexA− mutL−          |
| RW720              | recA− lexA− mutL−          |
| RW740              | recA− lexA− dnaE486 mutL   |
| RW666              | recA− lexA− mutL-pCJ102    |
| RW742              | recA− lexA− ΔpolA+kan mutL218:ΔTn10pCJ102 |
| RW722              | recA− lexA51(Def) mutL211:ΔTn10pCJ102 |
| RW769              | recA730 lexA51(Def) mutL211:ΔTn10pCJ102 |
| RW784              | recA730 lexA51(Def) Δ(umuDC)596:ermGT mutL211:ΔTn10pCJ102 |
| RW704              | recA730 lexA51(Def) Δ(umuDC)596:ermGT mutL211:ΔTn10pCJ102 |
| RW708              | recA730 lexA51(Def) ΔdinB61::ble mutL211:ΔTn10pCJ102 |
| RW714              | recA730 lexA51(Def) ΔpolB−ΔSpc mutL211:ΔTn10pCJ102 |
| RW716              | recA730 lexA51(Def) ΔpolB−ΔSpc mutL211:ΔTn10pCJ102 |
| RW718              | recA730 lexA51(Def) Δ(umuDC)596:ermGT ΔdinB61::ble mutL211:ΔTn10pCJ102 |
|                   |                            |
| a. thr-1 araD139 Δ(gpt-proA)62 lacY1 tsx-33 glnV44 gaiK2 hisG4 rpsL31 ylt-5 mtl-1 argE3 thi-1 sulA211. |
| b. sulA211 thi-1 Δ(lac-pro)5 ilv(Ta) mtl-1 rpsL31 supD43. |
| c. pCJ102 = F′5′→w exonuclease of pol I, CmR (Joyce and Grindley, 1984).

### Table 2. Mutation rate for missense mutations in the rpoB gene of various E. coli strains.

| Strain designation | Relevant genotype/phenotype | Mutation rate (x10−9) | Confidence limits (95%) (x10−9) |
|--------------------|-----------------------------|------------------------|-------------------------------|
| RW118              | recA− lexA− mutL−          | 0.08                   | 0.07–0.11                     |
| RW720              | recA− lexA− mutL−          | 16.7                   | 15.9–18.6                     |
| RW740              | recA− lexA− dnaE486 mutL   | 34.2                   | 26.3–41.7                     |
| RW666              | recA− lexA− mutL-pCJ102    | 6.2                    | 4.7–8.3                       |
| RW742              | recA− lexA− ΔpolA+kan mutL218:ΔTn10pCJ102 | 3.8 | 3.4–5.4 |
| RW722              | recA− lexA51(Def) mutL     | 11.0                   | 9.8–12.7                      |
| RW690              | recA730 lexA51(Def) mutL   | 11.5                   | 9.8–16.9                      |
| RW694              | recA730 lexA51(Def) ΔumuDC mutL | 10.9 | 8.3–12.2 |
| RW708              | recA730 lexA51(Def) ΔdinB mutL | 13.6 | 11.2–15.2 |
| RW712              | recA730 lexA51(Def) ΔpolB mutL | 12.7 | 11.4–18.4 |
| RW710              | recA730 lexA51(Def) ΔumuDC ΔdinB mutL | 9.8 | 8.3–13.7 |
| RW714              | recA730 lexA51(Def) ΔpolB mutL | 17.4 | 13.8–20.6 |
| RW716              | recA730 lexA51(Def) ΔdinB ΔpolB mutL | 8.8 | 8.1–11.5 |
| RW718              | recA730 lexA51(Def) ΔumuDC ΔdinB ΔpolB mutL | 9.7 | 8.6–11.6 |
| RW604              | recA730 lexA51(Def) ΔumuDC mutL expressing UmuDC/C | 8.5 | 6.5–9.7 |
| RW604(pRW154)      | recA730 lexA51(Def) ΔumuDC mutL expressing UmuDC/C | 34.2 | 27.0–43.2 |
| RW604(pRW144)      | recA730 lexA51(Def) ΔumuDC mutL expressing MuCA(B) | 45.0 | 37.3–50.3 |
| RW604(pRW290)      | recA730 lexA51(Def) ΔumuDC mutL expressing RumaB | 46.3 | 24.3–72.0 |

a. The rpoB mutation rates were calculated using the Jones method of the median (Jones et al., 1994) applied to 29–40 individual cultures, and by using the equation, \( \mu = \frac{n}{2N} \) where \( \mu \) is the mutation rate per generation, \( n \) is number of mutations per culture and \( N \) is the final number of cells in the culture (Armitage, 1952).
located at positions 1532, 1534 and 1547 and a CG→TA transition at position 1546 (Table S1).

It is generally assumed that most genome duplication is performed by pol III holoenzyme with the help of pol I to seal Okazaki fragments (Kornberg and Baker, 1992). However, as noted in the introduction, both pol II and pol IV are expressed at reasonably high basal concentrations in an uninduced cell, so it is conceivable that both polymerases might contribute to spontaneous mutagenesis in wild-type cells. We were therefore interested in determining whether increasing the intracellular concentration of pols II and IV alters the spectrum of spontaneously arising rpoB mutations. To do so, we assayed rpoB mutagenesis in a lexA(Def) mutL strain (Fig. 1B), in which pols II and IV are expressed at maximally derepressed levels. Although both UmuD and UmuC are similarly expressed at high levels, the level of UmuD' is very low, so the amount of active pol V under these conditions is negligible (Woodgate and Ennis, 1991). In general, the spectra of rpoB mutations in the recA^+ lexA(Def) mutL strain were very similar to the recA^+ lexA^+ mutL strain, with the exception that there was one additional AT→GC hot spot at position 1552.

Next, we determined the spectra of rpoB mutations in a recA730 lexA51(Def) mutL strain. In this background, all three LexA-regulated polymerases are fully expressed and the mutant RecA730 protein efficiently converts

**Fig. 1.** Spectrum of spontaneous rpoB mutations arising in various mutL strains. CG→TA transitions are coloured green; CG→AT transversion are in pink; CG→GC transversions are in dark blue; AT→CG transversions are in red; AT→TA transversions are in turquoise; and AT→GC transitions are in yellow.

A. recA^+ lexA^+ mutL (RW720).
B. recA^+ lexA51(Def) mutL (RW722).
C. recA730 lexA51(Def) mutL (RW690).
Types of base-pair substitutions generated in rpoB in recA⁻ lexA⁻ mutL211::Tn5, recA⁻ lexA51(Def) mutL211::Tn5 and recA730 lexA51(Def) mutL211::Tn5 strains.

| Base-pair substitution | recA⁻ lexA⁻ mutL | recA⁻ lexA51(Def) mutL | recA730 lexA51(Def) mutL |
|------------------------|-------------------|------------------------|-------------------------|
| AT→TA                 | 6                 | 9                      | 20                      |
| AT→CG                 | 0                 | 0                      | 0                       |
| AT→GC                 | 335               | 293                    | 236                     |
| CG→GC                 | 0                 | 2                      | 1                       |
| CG→AT                 | 3                 | 3                      | 35                      |
| CG→TA                 | 27                | 63                     | 96                      |

UmuD to UmuD' in the absence of DNA damage (Shinagawa et al., 1988; Woodgate and Ennis, 1991), thereby activating pol V (Reuven et al., 1999; Tang et al., 1999). Interestingly, there was a noticeable difference in the rpoB spectra obtained in this background compared with either the recA⁻ lexA⁻ mutL or recA⁻ lexA51(Def) mutL backgrounds. In particular, there was a sharp drop in the number of AT→GC transition mutations at position 1547, as well as the appearance of multiple transversion mutations throughout the target region (Table 3), with the most notable being the CG→AT transversion at position 1576 (Table S1). As the transversion mutations were not observed in the recA⁻ lexA51(Def) mutL strain expressing pols I–IV, we hypothesize that the transversion events are most likely attributable to the mutagenic activity of pol V. Indeed, our observations are in good agreement with earlier studies showing that the pol V-dependent SOS mutator effect is characterized by a strong increase in transversion specificity (Yatagai et al., 1991; Miller and Low, 1984; Fijalkowska et al., 1997; Watanabe-Akanuma et al., 1997).

Low-level expression of pol V is the limiting factor influencing transversion mutations

When maximally expressed in a recA730 lexA51(Def) background, it is estimated that there are ~700 molecules of pol V per cell (Woodgate and Ennis, 1991), largely because the RecA730 protein efficiently mediates conversion of UmuD to UmuD', thereby circumventing both Lon- and ClpXP-degradation of UmuD (Frank et al., 1996). Under these conditions, levels of pol V are estimated to be approximately twofold higher than pol II (Bonner et al., 1988; Qiu and Goodman, 1997) and approximately three- to fourfold lower than pol IV (Kim et al., 2001). We were therefore interested in determining the effect of modest overproduction of pol V and the phylogenetically related pol R1 (MucA'B) and pol V₃₉₁ (RumA'B) on the spectrum of rpoB mutations in a ΔumuDC lexA51(Def) recA730 mutL background. To this end, E. coli UmuDC, R46/pKM101 MucAB and R391 RumAB were expressed from the low-copy-number plasmid, pGB2 (Churchward et al., 1984). Interestingly, modest overproduction of the pol V-like enzymes in the recA730 lexA51(Def) ΔumuDC mutL strain resulted in an ~4- to 5.5-fold increase in the overall mutation rate compared with the recA730 lexA51(Def) mutL strain (Table 2) and gave a very different distribution of spontaneous mutations, with the appearance of numerous hot spots and a substantial increase in the number of transversions (Fig. 2, Table 4, Table S2). Indeed, in the strain overexpressing E. coli pol V (UmuD'C), transversions accounted for ~34% of the total base substitutions assayed. The spectrum was dominated by nine hot spots, among which CG→AT transversions were located at positions 1576, 1592 and AT→TA transversions at position 1714. Overexpression of pol R1 (R46/pKM101 MucA'B) also resulted in 32 additional mutation sites and a higher increase of the number of transversions, accounting for 51% of the total number of base substitutions. CG→AT transversions hot spots were recovered at positions 1535, 1576, 1592 and AT→TA transversions at positions 1538, 1547, 1577, 1598 and 1714. Similarly, overexpression of pol V₃₉₁ (R391 RumA'B) also caused a dramatic increase in the number of transversions, representing 10 of the 13 hot spots at positions 1535, 1537, 1546, 1565, 1576, 1577, 1592, 1598, 1714 and 1715. Comparison of the data presented in Fig. 1C with that of Fig. 2 therefore suggests that chromosomally encoded levels of pol V are the limiting factor for the production of spontaneous transversion mutations. This is evidenced by the fact that modest overproduction of pol V-like enzymes causes a dramatic increase in transversion hot spots, especially CG→AT mutations at positions 1535, 1576, 1598, 1691 and AT→TA mutations at positions 1598 and 1714. In general, the spectra of rpoB mutations generated in the presence of overproduced pol V-like enzymes was similar (Table S2), but there were also polymerase-specific hot spots, such as the pol R1-dependent AT→TA transversion at position 1538 and the pol V₃₉₁-dependent CG→AT transversion at position 1565.

Competition between E. coli’s polymerases in a recA730 lexA51(Def) background

The data presented in Figs 1 and 2 suggest that pol V is responsible for most of the transversion mutations recovered in rpoB in a lexA51(Def) recA730 mutL background. To confirm this observation, we analysed the spectra of rpoB mutations in a ΔumuDC lexA51(Def) recA730 mutL strain (Fig. 3B, Table 5, Table S3). As expected, in the absence of pol V, the number of AT→TA and CG→AT transversions dropped significantly. As controls for these experiments, we also determined the spectra of rpoB mutations
Fig. 2. Spectrum of spontaneous \textit{rpoB} mutations arising in \textit{mutL} strains moderately overexpressing polV-like polymerases. CG\(\rightarrow\)TA transitions are coloured green; CG\(\rightarrow\)AT transversion are in pink; CG\(\rightarrow\)GC transversions are in dark blue; AT\(\rightarrow\)CG transversions are in red; AT\(\rightarrow\)TA transversions are in turquoise; and AT\(\rightarrow\)GC transitions are in yellow.

A. \textit{recA730 lexA51(Def) \Delta(umuDC) mutL (RW604)}.
B. \textit{recA730 lexA51(Def) \Delta(umuDC) mutL / pUmuDC}.
C. \textit{recA730 lexA51(Def) \Delta(umuDC) mutL / pMucAB}.
D. \textit{recA730 lexA51(Def) \Delta(umuDC) mutL / pRumAB}.

Data for RW604 and RW604/pRW290 were taken from Mead \textit{et al.} (2007) and are shown for comparison.
recovered from a $\Delta$dinB lexA(Def) recA730 mutL and $\Delta$polB lexA(Def) recA730 mutL strain (Fig. 3C and D). Interestingly, in the $\Delta$dinB lexA(Def) recA730 mutL strain there was an increase in AT→GC transitions at position 1547, as well as a dramatic decrease in the overall number of CG→AT transversions (Table 5). The latter suggests that in addition to pol V, pol IV is also involved in the generation of transversion mutations. Such observations are in agreement with Kuban et al. who reported that both pol IV and pol V are required to produce lagging-strand transversions in a lacZ reversion assay (Kuban et al., 2006). The spectrum of rpoB mutations recovered from the $\Delta$polB lexA(Def) recA730 mutL strain was very similar to the isogenic polB + strain. The major exceptions were a reduction in the GC→TA transition hot spot at position 1546 and a concomitant increase in the AT→GC transition hot spot at position 1547, and an increase in AT→TA transversions at position 1714.

Our observation that both pol IV and pol V may function together to promote transversions, and that pol II may also influence spontaneous mutagenesis at certain sites prompted us to analyse the spectra of rpoB mutations generated in a recA730 lexA(Def) mutL background in the absence of pols II, IV and V in various combinations (Fig. 4, Table 5 and Table S3). Indeed, in the absence of pol IV and pol V all of the CG→AT transversions disappeared, and only five AT→TA transversions were recovered at position 1714, thereby confirming that pol IV and V work in a common pathway to promote transversion mutations.

The recA730 lexA(Def) $\Delta$polB $\Delta$umuDC mutL strain exhibited the same general spectrum as the recA730 lexA(Def) $\Delta$umuDC mutL strain alone (cf. Fig. 3B versus Fig. 4C). Similarly, the recA730 lexA(Def) $\Delta$polB $\Delta$dinB mutL double mutant gave a spectrum of rpoB mutations that was similar to the strain carrying a deletion of dinB alone (cf. Fig. 3C versus Fig. 4D), but which was dominated by an increase in the number of AT→GC transition mutations at position 1547. Thus, it appears that pols II and IV act to suppress transition mutations at this location. Interestingly, in the recA730 lexA(Def) mutL strain lacking pols II, IV and V, the number of AT→GC transition mutations at position 1547 drops considerably (Fig. 4E). As a significant number of AT→GC transitions at position 1547 were observed in the recA730 lexA(Def) $\Delta$umuDC mutL strain (Fig. 4B), they are clearly not dependent upon pol V, but rather appear to be modulated by its presence. Last, but not least, the spectrum of mutations

| Base-pair substitution | recA730 lexA51(Def) $\Delta$umuDC mutL | recA730 lexA51(Def) $\Delta$umuDC mutL pUmuDC | recA730 lexA51(Def) $\Delta$umuDC mutL pMucAB | recA730 lexA51(Def) $\Delta$umuDC mutL pRumAB |
|------------------------|----------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|
| AT→TA                 | 4                                      | 67                                         | 118                                         | 55                                         |
| AT→CG                 | 1                                      | 5                                         | 18                                          | 13                                          |
| AT→GC                 | 248                                    | 142                                        | 93                                          | 101                                         |
| CG→GC                 | 0                                      | 1                                         | 8                                           | 9                                           |
| CG→AT                 | 0                                      | 52                                         | 51                                          | 94                                          |
| CG→TA                 | 93                                     | 102                                        | 85                                          | 63                                          |

Table 5. Types of base-pair substitutions generated in rpoB in recA730 lexA(Def) mutL21f::Tn5 strains lacking pol II, pol IV or pol V alone, or in combination.

| Base-pair substitution | recA730* lexA(Def) mutL | recA730* lexA(Def) $\Delta$umuDC mutL | recA730* lexA(Def) $\Delta$dinB mutL | recA730* lexA(Def) $\Delta$polB $\Delta$umuDC mutL |
|------------------------|--------------------------|----------------------------------------|--------------------------------------|-----------------------------------------------|
| AT→TA                 | 20                       | 4                                      | 11                                   | 26                                           |
| AT→CG                 | 0                        | 0                                      | 2                                    | 5                                            |
| AT→GC                 | 236                      | 303                                    | 279                                  | 265                                          |
| CG→GC                 | 1                        | 0                                      | 3                                    | 0                                            |
| CG→AT                 | 35                       | 3                                      | 2                                    | 24                                           |
| CG→TA                 | 96                       | 58                                     | 59                                   | 45                                           |

a. Data taken from Table 3 and shown for comparison.
Fig. 3. Spectrum of spontaneous $rpoB$ mutations arising in $mutL$ strains lacking pols II, IV or V. CG→TA transitions are coloured green; CG→AT transversion are in pink; CG→GC transversions are in dark blue; AT→CG transversions are in red; AT→TA transversions are in turquoise; and AT→GC transitions are in yellow.

A. recA730 lexA51(Def) $mutL$ (RW690).
B. recA730 lexA51(Def) Δ(umuDC) $mutL$ (RW694).
C. recA730 lexA51(Def) ΔdinB $mutL$ (RW708).
D. recA730 lexA51(Def) ΔpolB $mutL$ (RW712). The data presented for RW690 are the identical to that reported in Fig. 1C and are shown for comparison.
Fig. 4. Spectrum of spontaneous rpoB mutations arising in mutL strains lacking a combination of pols II, IV or V. CG→TA transitions are coloured green; CG→AT transversion are in pink; CG→GC transversions are in dark blue; AT→CG transversions are in red; AT→TA transversions are in turquoise; and AT→GC transitions are in yellow.

A. recA730 lexA51(Def) mutL (RW690).
B. recA730 lexA51(Def) ΔumuDC ΔdinB mutL (RW710).
C. recA730 lexA51(Def) ΔumuDC ΔpolB mutL (RW714).
D. recA730 lexA51(Def) ΔpolB ΔdinB mutL (RW716).
E. recA730 lexA51(Def) ΔpolB ΔdinB ΔumuDC mutL (RW718). The data presented for RW690 are the identical to that reported in Fig. 1C and are shown for comparison.
recovered from the recA730 lexA(Def) ΔpolB, ΔdinB, ΔumuDC mutL strain (Fig. 4E) must reflect errors generated by either pol I or pol III, as they are the only known polymerases remaining in the triple deletion strain. The distribution of mutations was dominated by AT→GC transition hot spots at positions 1532, 1534, 1538, 1547 and 1552 and CG→TA transitions at positions 1546, 1576 and 1671. This spectrum largely resembled that observed in a recA+ lexA(Def) mutL background (cf. Fig. 1B versus Fig. 4E). From this, we can infer that pols II, IV or V do not promote rpoB mutagenesis in a recA+ lexA(Def) mutL strain and as a consequence, most spontaneous rpoB mutagenesis observed in a recA+ lexA(Def) mutL strain is dependent upon pols I and III.

**Role of pol I and pol III in spontaneous mutagenesis**

The data presented in Fig. 4E indicate that in a recA730 lexA(Def) mutL background, pol I or pol III is error-prone at particular hot spots within rpoB. To try and determine the relative contribution of each polymerase to spontaneous mutagenesis, we considered making recA730 lexA(Def) mutL strains with mutations in either polA or dnaE (encoding the catalytic α-subunit of pol III holoenzyme). However, polA recA mutants are inviable (Gross et al., 1971; Fijalkowska et al., 1989; Witkin and Roegner-Maniscalco, 1992) and dnaE recA730 mutL strains have very low viability (Fijalkowska et al., 1997). As a consequence, we analysed the effects of polA and dnaE in a recA+ lexA+ mutL background, where we have deduced that the four major hot spots in rpoB are largely attributed to misincorporation by pol I and/or pol III (cf. Fig. 1A versus Fig. 4E).

To analyse the role of pol III on rpoB mutagenesis we used dnaE486. This is a temperature-sensitive missense allele of dnaE caused by an S885P mutation (Vandewiele et al., 2002). This residue is not located close to the catalytic active site of the α-subunit, but presumably affects the ability of the α-subunit to interact with other subunits of the holoenzyme complex (Lamers et al., 2006). While dnaE486 strains exhibit temperature-sensitive growth and are inviable at 43°C, they are able to grow at 37°C and at this temperature they exhibit a moderate mutator phenotype (Wechsler and Gross, 1971; Fijalkowska et al., 1997). This mutator phenotype is largely dependent upon pol V (Vandewiele et al., 2002), suggesting that pol V has greater access to genomic DNA in the dnaE486 background.

Interestingly, the recA+ lexA+ dnaE486 mutL spectrum was very different to that of the recA+ lexA+ mutL control (Fig. 5, Table 6, Table S4). There was a significant decrease in AT→GC transitions at positions 1532 and 1534, suggesting that these hot spots could be pol III-dependent. Conversely, there was an increase in transversion mutations when compared with the isogenic dnaE+ strain (Table 6). In particular, we observed an increase in CG→TA transversions at position 1576, which we previously demonstrated are dependent upon pol IV and pol V (Figs 2A, B and 3B, Table S4).

ΔpolA strains are inviable when grown in rich media unless the strain carries a plasmid either expressing the 5′→3′ or 3′→5′ exo(exonuclease) domain of the polymerase (Joyce and Grindley, 1984). In our case, we used a strain carrying pCJ102, which expresses the 5′→3′ exonuclease of pol I (Joyce and Grindley, 1984). We first introduced the plasmid into the recA+ lexA+ mutL strain and assayed the spectrum of mutations in rpoB (Fig. 6, Table 7, Table S5). Somewhat surprisingly, we observed a significant decrease in the number of AT→GC transitions at position 1547 compared with the isogenic strain lacking pCJ102, as well as slight increases in the number of transitions at positions 1534 and 1546. Interestingly, deletion of pol I added to these effects in that there was a further decrease in the number of AT→GC transitions recovered at position 1547, and a concomitant increase in AT→GC and CG→TA transitions at positions 1534 and 1546 respectively. Thus, three of the four major transition hot spots within rpoB appear to be modulated by pol I, but in opposite ways. Pol I appears to suppress transition mutations arising at positions 1534 and 1546, but is largely responsible for the AT→GC transition mutation occurring at position 1547. These mutations presumably occur when pol I seals Okazaki fragments during genome duplication, or during the postreplicative gap-filling step of nucleotide excision repair. As noted above (Fig. 4), AT→GC transitions mutations at position 1547 increase significantly in the absence of pols II and IV. Thus, it appears that at this particular site, pols II and IV work together to suppress pol I-dependent misincorporation events.

**Discussion**

The multiplicity of pols in *E. coli* requires that access of each polymerase to chromosomal DNA must be exquisitely regulated. To gain insights into the interplay that takes place among *E. coli*’s five pols, we have exploited the fact that each polymerase leaves a distinct ‘genetic fingerprint’ when copying DNA (Wolff et al., 2004). We analysed the spectrum of rpoB mutants generated in a set of 13 isogenic mutL strains. The use of strains defective in mismatch repair was imperative, as it allowed us to follow polymerase-specific misincorporation events, rather than assay mutations that escape repair. The strains also carried mutations in recA and lexA that lead to the differential expression of *E. coli*’s three SOS-regulated pols, pols II, IV and V. The recA+ lexA+ mutL strain served as the baseline for spontaneous mutagenesis in the absence
of any SOS induction. In contrast, the recA\(^{+}\) lexA\(^{+}\) mutL strain allowed us to determine the effects of pols II and IV when expressed at fully derepressed levels, and the recA730 lexA\(^{+}\) mutL strain, in which pol V is maximally activated, allowed us to investigate the potential competition between all five of \textit{E. coli}'s pols. Furthermore, although we used genetically modified strains to derepress expression of pols II, IV and V, we believe that our observations can be extrapolated to wild-type \textit{E. coli} cells growing under stressful conditions, where there is likely to be transient upregulation of pols II, IV and V.

Given that it is well known that mismatch repair largely protects an organism against transition mutations and has little effect on transversions (Schaaper and Dunn, 1987; 1993), it was no surprise that in our \textit{rpoB} assays, the spectrum of mutations observed in the recA\(^{+}\) lexA\(^{+}\) mutL strain was dominated by AT\(\rightarrow\)GC and CG\(\rightarrow\)TA transitions at four well-defined hot spots (1532, 1534, 1546 and 1564).

### Table 6. Types of base-pair substitutions generated in \textit{rpoB} in recA\(^{+}\) lexA\(^{+}\) dnaE mutL and recA\(^{+}\) lexA\(^{+}\) dnaE486 mutL strains.

| Base-pair substitution | recA\(^{+}\) lexA\(^{+}\) dnaE mutL | recA\(^{+}\) lexA\(^{+}\) dnaE486 mutL |
|------------------------|-------------------------------|---------------------------------|
| AT\(\rightarrow\)TA     | 6                             | 13                              |
| AT\(\rightarrow\)CG     | 0                             | 0                               |
| AT\(\rightarrow\)GC     | 335                           | 229                             |
| CG\(\rightarrow\)GC     | 0                             | 1                               |
| CG\(\rightarrow\)AT     | 1                             | 34                              |
| CG\(\rightarrow\)TA     | 27                            | 109                             |

\(^{a}\) Data taken from Table 3 and shown for comparison.

### Table 7. Types of base-pair substitutions generated in \textit{rpoB} in recA\(^{+}\) lexA\(^{+}\) dnaE\(\rightarrow\) mutL211::Tn5, recA\(^{+}\) lexA\(^{+}\) dnaE486 mutL211::Tn5 and recA\(^{+}\) lexA\(^{+}\) D\(\rightarrow\)polA mutL218::Tn10/pCJ102.

| Base-pair substitution | recA\(^{+}\) lexA\(^{+}\) mutL \(\rightarrow\) pCJ102\(^{b}\) |
|------------------------|---------------------------------|
| AT\(\rightarrow\)TA     | 6                               | 21                              |
| AT\(\rightarrow\)CG     | 0                               | 0                               |
| AT\(\rightarrow\)GC     | 335                             | 250                             |
| CG\(\rightarrow\)GC     | 0                               | 0                               |
| CG\(\rightarrow\)AT     | 1                               | 2                               |
| CG\(\rightarrow\)TA     | 27                              | 117                             |

\(^{a}\) Data taken from Table 3 and shown for comparison.

\(^{b}\) pCJ102 = F\(\rightarrow\)S\(\rightarrow\)S\(\rightarrow\) exonuclease of pol I, Cm\(^{R}\) (Joyce and Grindley, 1984).
There was little difference in the spectrum of rpoB mutations recovered from the recA<sup>+</sup> lexA<sup>+</sup> mutL and recA<sup>+</sup> lexA<sup>+</sup> (Def) mutL strains, suggesting that even when fully derepressed, pols II and IV are unable to compete with pol I and/or pol III for access to a nascent chromosomal primer. In contrast, in the recA730 lexA(Def) mutL strain, there was a dramatic change in the mutagenic spectrum with a shift from transition to transversion mutations.

Thus, when fully derepressed, and maximally activated, it appears that pols II, IV and V are able to compete with pols I and III for access to genomic DNA and in doing so, either suppress or promote mutagenesis on the E. coli chromosome. For example, one of the most notable mutagenic events observed in rpoB was a dramatic increase in transitions at position 1547 in the recA730 lexA(Def) ΔpolA mutL/pCJ102 (F<sup>′</sup> 5′-3′ pol I exonuclease) strain. By comparing rpoB mutagenesis in a recA<sup>+</sup> lexA<sup>+</sup> mutL strain to an isogenic strain carrying a deletion of polA, we determined that pol I is, in fact, responsible for most transition mutagenesis at position 1547. Such observations are remarkable given that pol I possesses both 3′→5′ and 5′→3′ exonuclease activities and is generally thought to

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**Fig. 6.** Effect of deleting polA on the spectrum of spontaneous rpoB mutations arising in recA<sup>+</sup> lexA<sup>+</sup> mutL strains. CG→TA transitions are coloured green; CG→AT transversion are in pink; CG→GC transversions are in dark blue; AT→CG transversions are in red; AT→TA transversions are in turquoise; and AT→GC transitions are in yellow.

A. recA<sup>+</sup> lexA<sup>+</sup> mutL (RW720).  
B. recA<sup>+</sup> lexA<sup>+</sup> mutL/pCJ102 (F<sup>′</sup> 5′-3′ pol I exonuclease) (RW766).  
C. recA<sup>+</sup> lexA<sup>+</sup> ΔpolA mutL/pCJ102 (F<sup>′</sup> 5′-3′ pol I exonuclease) (RW742). The data presented for strain RW720 are identical to that reported in Fig. 1A and are shown for comparison.
be an accurate polymerase, with misincorporations occurring with a frequency of $< 1 \times 10^{-6}$ (Bebenek et al., 1990). The fact that mutagenesis at position 1547 increases in a ring with a frequency of $10^{-6}$ for pol II (Banach-Orlowska et al., 2005; Gawel et al., 2008). However, pol IV lacks intrinsic exonuclease activity and presumably acts as a simple competitive inhibitor to prevent access to a primer terminus by error-prone pol I.

In contrast to the transition mutations, our study revealed that transversion mutations in a recA730 lexA(Def) mutL strain are largely dependent upon the combined actions of pol IV and pol V and that cellular levels of pol V are limiting for transversion mutagenesis.

The initial goal when embarking upon this study was that we would identify which particular DNA polymerase is responsible for specific mutagenic events within rpoB. Indeed, the AT→GC transition mutations observed at position 1547 and the CG→AT transversions at position 1576 are generated through the mutagenic actions of pol I and pol IV/V respectively. In contrast, pol II appears to play a largely antimutagenic role by suppressing mutagenesis at certain hot spots. Until now, it has tacitly been assumed that spontaneous mutations occurring in the E. coli genome largely arise during genome duplication performed by the cell’s replicase, pol III. This may be true for the transition mutations recovered at positions 1534 and 1546. However, based upon our observations above, we also have to conclude that under certain conditions, there is considerable switching among E. coli’s five pols and that such interplay modulates the extent of spontaneous mutagenesis occurring on the E. coli chromosome, especially in cells growing under stressful conditions.

**Experimental procedures**

**Bacterial strains and plasmids**

Most of the E. coli K-12 strains used in this study are derivatives of RW118 (full genotype: thr-1 araD139 Δ(gpt-proA)62 lacY1 tsx-33 supE44 galK2 hisG4 rpsL31 xyl-5 mtl-1 argE3 thi-1 sulA211) (Ho et al., 1993). The exception was strain RW604 [full genotype: thi-1 Δ(lac-gpt)6 lvm(Ts) mtl-1 rpsL31 supD43 recA730 srfC300::Tn10 lexA(Def) sulA211 ΔumuDC595::cat mutL11::Tn5] (Mead et al., 2007) (Table 1). All derivatives of RW118 were made by standard methods of P1 transduction using P1vir (Table 1). The various polymerase or mutL alleles used in this study were obtained from the following strains: CJ231 for ΔpolA::kan (Joyce and Grindley, 1984); STL1366 for ΔpolB::spec (Rangarajan et al., 1999); AR30 for ΔdinB61::ble (Borden et al., 2002); EC8 for ΔumuDC596::ermGT (Frank et al., 1996); RW620 for dnaE866 zae502::Tn10 (Rangarajan et al., 1999); NR10775 for mutL211::Tn5 (Schaaper, 1996); and ES1484 for mutL218::Tn10 (Siegel et al., 1982).

Low-copy-number plasmids derived from pGB2 (Churchward et al., 1984), which encode E. coli umuDC (pRW154), R46/pKM101 mucAB (pRW144) or R391 rumAB (pRW290), have been described previously (Ho et al., 1993; Szekeres et al., 1996; Mead et al., 2007).

Where noted, bacteria were grown on LB agar plates containing 20 μg ml⁻¹ chloramphenicol, 15 μg ml⁻¹ tetracycline, 25 μg ml⁻¹ zeocin, 50 μg ml⁻¹ kanamycin, 50 μg ml⁻¹ spectinomycin and 100 μg ml⁻¹ rifampicin.

**Colonies PCR assay to test for ΔpolA, ΔpolB, ΔdinB and ΔumuDC genotypes**

Although the ΔpolA, ΔpolB, ΔdinB and ΔumuDC substitution alleles are all marked with a selectable antibiotic resistance, we encountered situations where we obtained a large number of antibiotic-resistant colonies (especially with ΔumuDC::ermGT) that ultimately turned out to be false positives. As a consequence, all transductants were initially selected by growth on the appropriate antibiotic-containing media and subsequently confirmed by colony PCR.

The primers used to detect the ΔpolA::kan allele were POLA1 (5′-TTC CGA CCA TCA AGC ATT TTA T−3′) and POLA2 (5′-TCA GCC ATT AGC GAT CTT TTT T−3′). The temperature profile used with these primers was 25 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, which results in the amplification a 923 bp PCR fragment.

The primers used to detect the Δ(araD-polB)::Δ2 allele were SPCR2 (5′-TCT GTC CGT GCT GCC GAA CTA−3′) and POLB (5′-CCG AGC GGA TCA ATC AGA AAG GTG−3′). The temperature profile used with these primers was 25 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, which results in the amplification an 817 bp PCR fragment.

The primers used to detect the ΔdinB61::ble allele were AR270 (5′-GCC ATG ACC GAT GTC GCC GAG C−3′) and AR271 (5′-TGT ATA CTT TAC CAG TGT TGA GAG G−3′). The temperature profile used with these primers was 35 cycles of 95°C for 30 s, 60°C for 1 min and 72°C for 2 min, which results in the amplification a 318 bp PCR fragment.

The primers used to detect the ΔumuDC596::ermGT allele were EMR1 (5′-GCC CTA GTC TCA AAG ACA CTA−3′) and ERM2 (5′-CAC CCT TCA AAA ATA TCA CTC AAA−3′). The temperature profile used with these primers was 25 cycles at 94°C for 1 min, 50°C for 1 min and 72°C for 2 min, which results in the amplification a 1165 bp PCR fragment.

**Spectra of spontaneous mutations in rpoB**

The mutation spectra were generated using the rpoB/RifR mutagenesis assay developed by Jeffery Miller’s group (Garibyan et al., 2003; Wolff et al., 2004). The rpoB gene encodes the β-subunit of RNA polymerase and base-pair substitutions in rpoB are either lethal or result in rifampicin resistance. Eighty-eight per cent of all rpoB mutations are localized in the central 202 bp region of the gene (Garibyan et al., 2003), thus offering the advantage that a single pair of oligonucleotide primers can be used for PCR amplification, and a single primer for DNA sequencing (Mead et al., 2007). The protocol used to identify rpoB mutations in the various strain backgrounds is detailed below. Each strain was diluted...
from a frozen stock culture and plated on the appropriate antibiotic-containing Luria–Bertani (LB) agar plates in order to obtain at least 600 independent colonies. Plates were incubated at 37°C overnight. Single colonies were picked and used to inoculate ~600 independent LB cultures per strain. Cultures were grown for 24 h at 37°C and streaked on an LB agar plate containing 100 μg ml⁻¹ rifampicin. A single Rif⁺ colony was picked from each streak. To avoid bias based on colony size, the Rif⁺ colony closer to a predetermined target point was chosen from each streak. Colony PCR was then performed on individual colonies in a 96-well microtitre plate. An ~1 kb central region of the rpoB gene was amplified 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′. Amplification was achieved 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, followed by a final extension step at 72°C for 7 min. Primer WOG923AP01 (5′-CAG TTC CGC GTT GGC -CAG TTC CGC GTT GGC -3′) was used to determine the nucleotide sequence of the 300 bp target region of rpoB in each PCR amplicon (Cogenics, Houston, TX). Only base-pair substitutions occurring between positions 1516 and 1717 of the rpoB gene were considered during data analysis. Nucleotide sequences obtained were aligned and analysed using the SeqManager program of the DNASTAR suite (Madison, WI).

For derivatives of RW604 harbouring pRW154, pRW144 or pRW290, the protocol was modified slightly. Instead of initially diluting cultures from a frozen stock culture, RW604 was freshly transformed with plasmid DNA and ~600 individual transformants were immediately picked for subsequent rpoB analysis (Mead et al., 2007).

**Determination of mutation rates**

Each strain was diluted from a frozen stock culture into fresh liquid media to give an initial count of ~100 cells ml⁻¹ and grown overnight at 37°C. Aliquots (50 μl) of each overnight culture were plated on agar plates containing 100 μg ml⁻¹ of rifampicin for selection of the forward mutation in rpoB. Plates were incubated overnight at 37°C. The number of viable colonies in the culture was determined by simply plating 50 μl of the appropriate serial dilution (in PBS media) onto LB agar plates in duplicate, followed by overnight incubation at 37°C. For the wild-type mutL⁻ strain, RW118, 3 ml cultures were harvested by centrifugation and re-suspended in an equal volume of PBS. The rpoB mutation rates were calculated as recommended (Foster, 2006), using the Jones method of the median (Jones et al., 1994) applied to 29–40 individual cultures and using the equation, \( \mu = m/2Nt \), where \( \mu \) is the mutation rate per generation, \( m \) is number of mutations per culture and \( Nt \) is the final number of cells in the culture (Armitage, 1952).

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