Competitive Fitness during Feast and Famine: How SOS DNA Polymerases Influence Physiology and Evolution in *Escherichia coli*

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Running Head: SOS Polymerases, Survival and Evolution

Keywords: Stationary phase, GASP, SOS polymerase, translesion synthesis, alternative DNA polymerase, Pol II (*polB*), Pol IV (*dinB*), Pol V (*umuDC*), *E. coli*, chemostat

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Abstract

*Escherichia coli* DNA polymerases II, IV and V serve dual roles by facilitating efficient replication past DNA damage while simultaneously introducing genetic variation that can promote adaptive evolution. Here we show that these alternative polymerases are induced as cells transition from exponential to long-term stationary phase growth in the absence of SOS induction with external agents. By monitoring the relative fitness of isogenic mutant strains expressing only one alternative polymerase over time, spanning hours to weeks, we establish distinct growth phase-dependent hierarchies of polymerase mutant strain competitiveness. Pol II confers a significant physiological advantage by facilitating efficient replication and creating genetic diversity during periods of rapid growth. Pol IV and Pol V make the largest contributions to evolutionary fitness during long-term stationary phase. Consistent with their roles providing both a physiological and adaptive advantage during stationary phase, the expression patterns of all three SOS polymerases changes during the transition from log phase to long-term stationary phase. Compared to the alternative polymerases, Pol III transcription dominates during mid-exponential phase, however its abundance decreases to less than 20% during long-term stationary phase. Pol IV transcription dominates as cells transition out of exponential phase into stationary phase and a burst of Pol V transcription is observed as cells transition from death phase to long-term stationary phase. These changes in alternative DNA polymerase transcription occur in the absence of SOS induction by exogenous agents and indicate that cell populations require appropriate expression of all three alternative DNA polymerase during exponential, stationary and long-term stationary phases to attain optimal fitness and undergo adaptive evolution.
Introduction

The generation of genetic diversity directly impacts the evolutionary fitness of a population, and the molecular mechanisms influencing the formation of allelic variation often dictate success or failure within complex bacterial communities (BJEDOV et al. 2003; CHAO and COX 1983; PIGLIUCCI 2008; SAINT-RUF and MATIC 2006; WOODS et al. 2011). Given that the majority of mutations are introduced during replication, perturbations in the fidelity of replication can have dramatic consequences on the evolutionary trajectory of a population (FOSTER 2007; GALHARDO et al. 2007; KUNKEL 2004; YEISER et al. 2002). Accordingly, characterization of the mechanisms and extent to which DNA polymerases introduce genetic variation is critical to understanding the physiology and evolution of bacteria.

Escherichia coli encodes five DNA polymerases (FRIEDBERG 2006; JOHNSON and O’DONNELL 2005; Goodman 2002). High-fidelity DNA polymerase III performs the majority of DNA replication under vegetative conditions, with Pol I contributing principally to maturation of Okazaki fragments (FRIEDBERG 2006; KORNBERG and BAKER 1992). Three alternative DNA polymerases (Pol II, Pol IV and Pol V) perform a vital physiological role by mediating translesion synthesis (TLS), enabling efficient replication past DNA damage that would otherwise halt replication, albeit with significantly reduced fidelity (BICHARA et al. 2011; FUCHS et al. 2004; GOODMAN 2002; NOHMI 2006; TIPPIN et al. 2004). These error-prone DNA polymerases can be induced under a variety of environmental stresses (LAYTON and FOSTER 2003; LAYTON and FOSTER 2005; MACPHEE and AMBROSE 2010; STUMPF and FOSTER 2005; TADDEI et al. 1995; YEISER et al. 2002) and have been characterized most extensively following induction of the SOS regulon in response to DNA damage, leading them to be referred to as SOS-induced polymerases (COURCELLE et al. 2001; FRIEDBERG 2006; NOHMI 2006; YANG and...
WOODGATE 2007, Goodman 2002). DNA polymerase II (encoded by polB) is a B-family polymerase (BANACH-ORLOWSKA et al. 2005; PHAM et al. 2001; RANGARAJAN et al. 1999; RANGARAJAN et al. 2002) capable of 3’-exonuclease proofreading, enabling it to replicate undamaged DNA with considerable accuracy (CAI et al. 1995). DNA polymerases IV (dinB) and V (umuDC) are Y-family polymerases (GOODMAN 2002; NOHMI 2006; Ohmori et al. 2001) that replicate DNA with relatively lower fidelity (FUCHS et al. 2004; JAROSZ et al. 2007; TANG et al. 2000). Homologs of these alternative polymerases are found in all three domains of life (Ohmori et al. 2001), and have been implicated in a variety of human diseases (LANGE et al. 2011; ROBBINS et al. 1975; ROBBINS et al. 1974; STALLONS and Mcgregor 2010; XIE et al. 2010).

As a consequence of their physiological roles and comparatively low fidelity, considerable interest has focused on characterizing the impact of each alternative polymerase on the formation of genetic diversity, their impact on cell survival during stress, and the dynamics of polymerase competition for access to the replication fork (DELMAS and MATIC 2006; HASTINGS et al. 2010; NOHMI 2006; PETROSINO et al. 2009; SHEE et al. 2011; Yeiser et al. 2002). While most in vivo studies have focused on the roles of Pols II, IV and V either in the survival of rapidly dividing cells in the presence of artificial DNA damage sufficient to activate the E. coli SOS response or in a plate-based assay system studying the process of “adaptive mutation” (FOSTER 2005; FOSTER 2007; GALHARDO et al. 2007; ROTH et al. 2006), recent findings have also suggested growth conditions can influence which polymerases play greater roles in replication and alter the spectrum of mutations generated (NOWOSIELSKA et al. 2004).

Given the well-characterized roles of the alternative polymerases in the generation of adaptive mutations within these assays, numerous authors have speculated that the alternative
polymerases might serve to mediate stress-induced mutagenesis and influence the survival and evolution of bacterial populations. We have previously found (YEISER et al. 2002) that, in the absence of exogenous DNA damage, Pols II, IV and V provide a substantial fitness advantage to wild type cells during long-term stationary phase competition, including expression of the GASP (Growth Advantage in Stationary Phase) phenotype where mutants of increased fitness are isolated after long-term incubation in stationary phase (FINKEL 2006; ZAMBRANO et al. 1993). However, Yeiser et al. used strains with only single DNA polymerase gene deletions, making it impossible to assign specific activities to each enzyme.

Here we investigate the roles and relative contributions of Pols II, IV, and V to cell growth and evolutionary fitness using a series of isogenic mutant strains lacking each alternative polymerase in all possible combinations: as single null mutants, as combinations of double null mutants that express only one alternative polymerase (referred to in this paper as Pol II⁺-only, Pol IV⁺-only, and Pol V⁺-only), and as a triple null mutant. This study provides a comprehensive analysis of the expression patterns and the roles of Pols II, IV and V in the absence of exogenous DNA damage. We determine each polymerase’s contribution toward cell survival and evolutionary fitness during periods of feast, in exponential batch culture growth where nutrients are abundant, or in a chemostat where nutrients are continually being replenished, and periods of famine, as cells enter stationary phase and transition into long-term stationary phase where nutrients are being depleted. These data demonstrate significant evolutionary consequences and establish individual roles for each polymerase under conditions where different strains must compete to survive. We show that while any one of the polymerases can randomly generate a mutation that enables a cell to survive, there are nevertheless conditions during batch culture where the activity of each individual enzyme is either dominant or co-dominant, enabling the
prediction of evolutionary outcomes. While previous studies on the regulation of the alternative polymerases have focused on increased transcription in response to DNA damage from exogenous agents, here we observe major changes in the transcription pattern of each alternative polymerase throughout the five phases of the bacterial life cycle (FINKEL 2006) in the absence of induction by exogenous stressors, demonstrating a remarkable shift towards error-prone polymerase expression under stationary phase conditions.

Results

Pol IV and Pol V Confer Greater Relative Fitness than Pol II during Long-term Stationary Phase

When cultured individually in rich medium, log phase growth and stationary phase survival of all seven polymerase-deficient strains are indistinguishable from wild type (Figure S1). To determine whether strains capable of expressing only one alternative polymerase display altered fitness, double mutants were competed against one another in Type 1, initial low cell density, (Figure 1A-C) and Type 2, initial high density, (Figure 1E-G) long-term competition experiments. In Type 1 experiments both strains are co-inoculated at low initial population density (~10^6 CFU/ml), passing through lag and exponential phase, and co-cultured for two weeks. In Type 2 experiments, dense stationary phase cultures of each strain are mixed at high initial density (~10^9 CFU/ml), after which strains are co-incubated for two weeks.

In Type 1 competitions, initiated at low population density, most competitions (59%) ended as ties, with both strains reaching final densities within ten-fold of each other (Figure 1D). Among those competitions where one strain clearly dominates, the Pol V^+-only strain has an advantage over both the Pol IV^+-only and Pol II^+-only strains. However, in Type 2 competitions,
initiated with stationary phase populations at high cell density, there was a decisive winner in nearly all (93%) competitions (Figure 1H). Strains expressing only Pol II were significantly less fit than strains expressing only Pol IV (Figure 1E) or Pol V (Figure 1F), losing in 78% of competitions. In competitions between Pol IV\(^{-}\)-only and Pol V\(^{+}\)-only both strains performed equally well, with Pol IV\(^{-}\)-only winning as many competitions as Pol V\(^{+}\)-only (Figure 1G).

**Pol II Enables Cells to Express the GASP Phenotype Faster than Pol IV or Pol V**

To assess the capacity of each alternative polymerase to generate beneficial alleles, each double-mutant strain was subjected to GASP competition assays. In a typical GASP assay strains are aged for 10 days in monoculture to allow spontaneous random mutants to take over the population. Cells from these cultures are then introduced as a minority to a dense culture of unaged wild type cells (ZAMBRANO et al. 1993). After incubating in monoculture for ten days, all three aged mutant strains were able to outcompete the unaged wild type population when introduced as a minority (Figure 2A-C), indicating the presence of a beneficial GASP allele. However, the aged Pol II\(^{-}\)-only (Figure 2A) and Pol V\(^{+}\)-only (Figure 2C) populations drove unaged wild type populations to extinction faster than aged Pol IV\(^{-}\)-only (Figure 2B).

We also determined the GASP phenotype of each polymerase-deficient strain with respect to one another, rather than the wild type. Again, every aged population expressed the GASP phenotype over unaged populations (Figure 2D-I), however the strength of the GASP phenotype differed among strains as determined by the time it took for the minority population to take over the culture. For each competition, the day the minority became the majority was determined and the average day of takeover was calculated. The Pol II\(^{+}\)-only strain consistently
exhibited the GASP phenotype fastest, with an average time to takeover of 3.5 days, compared to
4.5 days or 5.8 days for the strains capable of expressing only Pol V or Pol IV, respectively.

Pol II Contributes to Relative Fitness More than Pol IV and Pol V during Serial Passage

Although the Pol II+-only strain was significantly less fit than Pol IV+-only and Pol V+-
only in Type 2 stationary phase competitions (Figure 1E-F), in Type 1 competitions initiated at
low cell densities, the Pol II+-only strain shows less of a competitive disadvantage (Figure 1A-
B). Pol II+-only cell yields are routinely 4 to 5 orders-of-magnitude lower than their competitors
in Type 2 competitions, but only 10-100 fold lower in Type 1 experiments. The critical
difference between these experiments is that in Type 1 competitions cells experience an
additional outgrowth, providing more opportunity to replicate and generate genotypic diversity
under nutrient-rich conditions. Since this additional outgrowth on the first day of competition
profoundly influenced the fitness of the Pol II+-only strain weeks later, we determined whether
subjecting each strain to five serial passages prior to competition would amplify this effect.

Following serial passage Pol II+-only populations were competed against unpassaged Pol
IV+-only (Fig. 3A-E) or Pol V+-only (Fig. 3F-J) to assess changes in fitness. Prior to the
passaging regimen, the unpassaged Pol II+-only populations never outcompete Pol IV+-only or
Pol V+-only strains in stationary phase competitions (Figure 3A&F). However, following each
additional passage the Pol II+-only populations displayed an increase in relative fitness, to the
extent that Pol II+-only populations passaged five times consistently outcompeted both Pol IV+-
only and Pol V+-only strains (Figure 3E&J). Furthermore, the Pol II+-only strain passaged five
times also performed better against similarly passaged Pol IV+-only and Pol V+-only strains,
compared to its performance prior to passaging (data not shown).
Pol II Confers Greater Relative Fitness than Pol IV or Pol V during Continuous Culture

To determine the relative physiological contributions of each polymerase under conditions promoting high rates of cell division, a series of competitions were performed under continuous culture conditions in a chemostat. The three strains encoding a single alternative polymerase were co-cultured in chemostats at increasing flow rates, corresponding to faster growth rates. In every competition Pol II+-only outperformed both Pol IV+-only and Pol V+-only (Figure 4), with average cell yields ~10% more at lower flow rates and as much as 250% more at higher flow rates.

Alternative Polymerases Affect Mutation Frequency and Spectrum

To elucidate the molecular basis of the differences in adaptive potential observed during long-term competition, we characterized the frequency and spectrum of spontaneous mutations in rpoB, encoding the β-subunit of RNA polymerase, conferring rifampicin resistance (RifR). Mutations known to confer drug resistance include all six classes of missense mutations, as well as in-frame amplifications and deletions (Garibyan et al. 2003; Jin and Gross 1988; Makiel-Dzbenska et al. 2011; Reynolds 2000; Severinov et al. 1994; Singer et al. 1993; Wolff et al. 2004; Wrande et al. 2008). 1272 independent cultures were assayed: 159 cultures each for the wild type, all three single-mutant, all three double-mutant, and the triple-mutant strains; significant strain-specific differences mutation frequencies were observed (Figures 5A-C). Among double-mutant strains, Pol V+-only had the greatest mutation frequency, significantly higher than Pol IV+-only, which in turn was significantly higher than that of Pol II+-only. Significant differences in mutation frequency distributions were determined using the K-S Test.
(Figures 5A&B), and average Rif$^R$ values also reflect these shifts in mutation frequency (Figure 5C). Among single-mutant strains, Pol IV$^{-}$ was similar to wildtype while Pol II$^{-}$ and Pol V$^{-}$ showed significantly lower mutation frequencies (Figure 5B&C). The triple-mutant strain showed an overall mutation frequency similar to the wild type.

We also assessed the spectrum of mutations generated in each strain by sequencing $rpoB$ in individual Rif$^R$ clones (Figure 5D). Out of 1009 sequenced isolates we identified 85 alleles across 54 nucleotide positions (Tables S1 and S2), with several alleles that appear to be previously unreported (indicated in Table S1). Strain-specific mutation spectrum differences were observed. The wild type and Pol V$^{+}$-only strains produced the greatest numbers of different Rif$^R$ alleles across the greatest number of nucleotide positions, while the strain deficient in all three alternative polymerases generated the fewest alleles at the fewest positions. Strain-specific mutation spectra are summarized in Figures 5D, with source data presented in Table S3. Wild type cells generated about half as many GC$\rightarrow$AT mutations compared to any other strain, but had more than twice as many GC$\rightarrow$CG mutations than all other strains except Pol V$^{+}$-only. Pol II$^{+}$-only generated more deletions than all other strains combined.

**Alternative Polymerase and Other SOS Genes are Induced During Stationary Phase**

Given their observed physiological and evolutionary impacts during periods of feast and famine in the absence of endogenous stressors, we determined the expression patterns for each alternative DNA polymerase during long-term incubation. Wild type cultures were sampled hourly for the first twelve hours and then every 24 to 48 hours over 14 days of long-term stationary phase incubation. Quantitative RT-PCR on total cellular RNA throughout long-term incubation (Figure 6A) revealed an induction of transcripts encoding all three alternative
polymerases (polB, dinB, umuDC), as well as the SOS response gene sulA. Relative to their abundance after 2 hours of exponential phase growth, by five hours, as the cells transition into stationary phase, the average abundance of transcripts encoding Pol II and Pol IV more than doubled (dinB, +2.2-fold; polB, +2.2-fold) and the catalytic subunit of Pol V was induced 50% by hour 7 (umuC, +1.5-fold). Meanwhile, after a modest initial increase, transcript levels for the dnaE-encoded catalytic subunit of Pol III decreased by more than 40% within 4 hours and the SOS response repressor lexA was reduced more than 30% within 5 hours. For comparison, expression levels of the early-exponential phase gene fis (BALL et al. 1992) peak at ~2 hours and decrease significantly from mid-logarithmic phase through stationary phase while expression of the stationary phase-specific dps gene (MARTINEZ and KOLTER 1997) increases from late log through stationary phase (data not shown).

When comparing the relative abundance of dnaE, polB, dinB, and umuC transcripts (Figure 6B) there is a dramatic decline in the average proportion of dnaE transcript from ~69% during logarithmic growth to 34% upon entry into stationary phase. By 24 hours the dinB transcript is most abundant (56%), significantly exceeding that of dnaE (31%). For most time points, there was a consistent hierarchy of transcript abundance among alternative polymerases, with dinB > polB > umuC. The induction of alternative polymerases corresponded with induction of sulA, an SOS gene, indicating that stationary phase conditions induce the SOS response in the absence of exogenous stressors. For comparison, induction of sulA during the SOS response after addition of the DNA-damaging agent mitomycin C was ~4-fold higher than the induction observed as cells transition into stationary phase in the absence of drug (data not shown).
Discussion

The long-term viability and evolutionary success of bacterial populations requires a balance between maintaining the capacity to replicate efficiently with high fidelity while simultaneously generating sufficient genetic diversity to facilitate adaptation and survival in changing environments. Our previous study, which showed that alternative DNA polymerases play a role in the evolutionary fitness of bacterial populations (Yeiser et al. 2002), left open the key issue of each enzyme’s physiological and mutagenic contribution. Here we show specific roles for each alternative DNA polymerase. During periods of rapid cell division Pol II contributes significantly to relative fitness by facilitating faster growth and the generation of genetic diversity, whereas Pol IV and Pol V introduce greater genetic variation, conferring increased relative fitness, under the more stressful conditions of long-term stationary phase. Furthermore, differences in the frequency and spectrum of allelic variation attributable to each polymerase suggest a competitive hierarchy for access to replicate DNA in vivo.

Inherent differences in expression patterns and polymerase fidelity influence the relative fitness of the various mutant strains, allowing predictable outcomes of experiments where the strains are engaged in head-to-head competition. Given that success during long-term stationary phase is influenced by the appearance of advantageous mutations, Type I and II competition experiments illustrate a race to generate beneficial alleles quickly (Figure 1). The observation that the double-mutant strains expressing only Pol IV or Pol V perform better than the Pol II−-only strain in both Type 1 and Type 2 competitions suggests that these polymerases play a greater role in creating mutations, including beneficial alleles, during long-term stationary phase. Consistent with the fact that Pol IV and Pol V have been shown to have inherently lower deoxynucleotide insertion fidelity and lack proofreading, we show these enzymes produce
genetic diversity more quickly than Pol II in slowly dividing, stationary phase populations. Since previous work has shown that many different types of mutations can confer the GASP phenotype (FARRELL & FINKEL 2003; FINKEL 2006; ZAMBRANO et al. 1993), the appearance of novel mutations conferring GASP serves as a proxy for the extent of mutational change that has occurred in the population.

Following a 10-day aging regimen where beneficial alleles are generated and selected, the Pol II+-only strain expressed the strongest GASP phenotype (Figure 2). This finding suggests that, after providing ample time for beneficial mutations to appear, Pol II provides a greater physiological contribution to overall fitness via increased replicative efficiency during growth than either Pol IV or Pol V, enabling cells to capitalize on beneficial GASP alleles more quickly. This role for Pol II is bolstered by our observation that the Pol II+ strain performed significantly better than the Pol IV+-only and Pol V+-only strains when experiments were initiated at low density (allowing additional outgrowth) and within chemostat competitions allowing continuous growth. The fact that serially passaged Pol II+ populations exhibit greater competitive fitness against passaged Pol IV+-only or Pol V+-only strains leads to the conclusion that Pol II not only plays an important physiological role with respect to the efficiency of DNA replication, but also contributes considerable genetic diversity during periods of rapid growth.

Previous work identified differences in the frequency and spectrum of mutations generated by alternative polymerases, however many of these studies analyzed either a limited subset of mutation classes, evaluated reporter genes on extra-chromosomal elements, looked at mutations generated under artificial expression conditions, followed treatment with exogenous stressors, or used strains with altered repair systems (CURTI et al. 2009; FOSTER 2005; FOSTER 2007; GALHARDO et al. 2007; HERSH et al. 2004; WOLFF et al. 2004). Here we looked at the
distribution of mutation frequencies under long-term laboratory growth conditions with endogenous expression in the absence of exogenous stressors, in wild type and isogenic mutant strains (Figure 5). The distribution of mutation frequencies reveals significant differences in the \textit{in vivo} fidelity of each polymerase. Use of the double-mutant strains enables the analysis of each polymerase’s contribution to overall mutation frequency. The Pol V$^+$-only strain was responsible for generating the greatest mutation frequency, followed by Pol IV$^+$-only, and Pol II$^+$-only. The hierarchy of fidelity we observe here is consistent with previously published \textit{in vitro} and \textit{in vivo} studies (CAI \textit{et al.} 1995; KOBAYASHI \textit{et al.} 2002; MAOR-SHOSHANI \textit{et al.} 2000; TANG \textit{et al.} 2000).

It is also important to compare the mutation frequency of the single-mutant strains where two polymerases can compete for access to replicate DNA (Figure 5), enabling additional inferences regarding their relative contributions when acting in concert. Here the Pol IV$^-$ strain exhibited a greater mutation frequency than either Pol II$^-$ or Pol V$^-$, which were approximately equal. Given that the relative abundance of each competing polymerase can influence which polymerase is most likely to gain access to the replication fork and that the Pol IV transcript was the most abundant under the conditions of our experiments, these findings suggest the presence or absence of Pol IV is the greatest overall determinant of mutation frequency. Pol IV confers an intermediate mutation frequency among strains capable of expressing only one alternative polymerase. When Pol II is absent, Pol IV appears to mask the lower fidelity of Pol V; when Pol V is absent, Pol IV may outcompete the higher fidelity polymerase Pol II.

In the strain lacking only Pol IV, leaving Pol II and Pol V to compete, the mutation frequency was similar to the strain expressing only Pol V, suggesting that Pol V might outcompete Pol II to replicate during long-term batch culture. Together, these findings are
consistent with a competitive hierarchy for access to replicate DNA in vivo of Pol IV > Pol V > Pol II when cells are incubating in long-term stationary phase. Therefore, even though Pol V is inherently more mutagenic, its impact on mutation frequency is modulated by Pol IV. Pol IV’s higher levels of expression enable it to outcompete the other polymerases and give it more opportunity to introduce genetic variation, consistent with its significant role in generating mutations in the lac system (Foster 2000; McKenzie et al. 2001). Surprisingly, the mutation frequency of the triple-mutant strain lacking all three SOS polymerases is similar to the wild type strain. This can potentially be explained if mutated cells that cannot achieve translesion synthesis are lost from the population and the surviving mutants that we detect are attributable to the basal error rate of the replicative Pol III itself. This may also account for the more restricted spectrum of mutations observed in the triple-mutant strain.

Among over 1000 spontaneous mutants resistant to rifampicin (see Table S1), we identified 85 alleles encompassing all six classes of missense mutations, as well as in-frame insertions and deletions. We found that the wild type and Pol V+-only strains generated the greatest numbers of different alleles across the greatest number of nucleotide positions in rpoB. The triple-mutant strain, completely deficient in alternative polymerase expression, introduced the least variety of alleles at the fewest positions, demonstrating the importance of these enzymes in creating genetic diversity.

Previous reports have identified “fingerprints” or mutational “hotspots” left by specific polymerases in various genetic backgrounds (Curti et al. 2009; Wolff et al. 2004). We also identified strain-specific fluctuations in the frequency of specific alleles and mutation classes. For example, nearly half as many GC→AT mutations were identified in the wild type strain compared to any other polymerase-deficient strain. Twice as many GC→CG mutations were
observed in wild type relative to all strains except Pol V+-only, suggesting Pol V plays an important role in generating these transversions. The number of deletions detected in the Pol II+-only strain was greater than all other strains combined. Since this strain is incapable of expressing Pol IV or Pol V, it suggests Pol II plays a significant role in generating deletions, as has been reported (HASTINGS et al. 2010; KOSKINIEMI and ANDERSSON 2009; RANGARAJAN et al. 1997; WANG and YANG 2009). While the alterations in mutation spectrum observed differ from polymerase fingerprints reported elsewhere, these discrepancies may reflect strain and environmental differences.

The fact that the alternative polymerases, members of the SOS regulon, are expressed in the absence of endogenous stressors during long-term stationary phase further illustrates their impact on the survival and evolution of bacterial populations. When replacing Pol III, these polymerases each play a central role in providing a balance that achieves rapid growth while generating sufficient mutations to ensure genetic diversity. The observation that each alternative polymerase contributes differently to the variety and quantity of mutations demonstrates the importance of competitive interactions among the polymerases. One explanation for the apparent increase in alternative polymerase expression during long-term stationary phase may be a positive selection on bacterial populations that increase genetic diversity during times of stress. Indeed, the simultaneous downregulation of dnaE and induction of alternative polymerase expression observed here could produce a shift towards stress-induced mutagenesis by shifting polymerase competition in favor of error-prone replication as has been proposed elsewhere (CURTI et al. 2009; FOSTER 2007; HASTINGS et al. 2010; SUTTON 2010).

It has long been appreciated that the alternative DNA polymerases can be induced following stress and influence the formation of adaptive mutations, yet the extent of their
contributions to long-term survival and evolution under conditions akin to those found in nature has remained primarily speculative. Our findings demonstrate specific physiological roles and evolutionary implications of each alternative DNA polymerase under conditions of both feast and famine. Given the ubiquitous nature of alternative DNA polymerases and their impact on cell fitness and survival, a deeper understanding of factors affecting their expression, competitive interactions, mutation preferences, and other cellular functions will yield valuable insights toward understanding the physiological responses and evolutionary trajectories of bacterial populations.

Materials and Methods

Strains Used and Mutant Construction

All strains (Table 1) are derived from *E. coli* K-12 strain ZK126 (W3110ΔlacU169 tna-2), including nalidixic acid-resistant parental strain ZK1142 (ZAMBRANO et al. 1993). DNA polymerase single, double, and triple mutants were constructed by bacteriophage P1 transduction into ZK126 using the following donor strains: for Pol II−, SH2101 (*polB::Spc*) (BONNER et al. 1992); for Pol IV−, RW626 (*dinB::Kan*); and for Pol V−, RW82 (*umuDC::Cam*) (both RW626 and RW82 were generous gifts from Roger Woodgate [National Institutes of Health, Bethesda, MD]). Genetic elements conferring antibiotic resistance are effectively neutral in the absence of drug selection (KRAIGSLEY and FINKEL 2009; YEISER et al. 2002). Strains lacking a single alternative polymerase are designated with a superscript “minus” sign (Pol II−, Pol IV−, and Pol V−), whereas double-mutant strains capable of expressing only one alternative polymerase are designated with a superscript “plus” sign coupled with “-only” (Pol II+/-only, Pol IV+/-only, and Pol V+/-only).
<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Genotype/Phenotype</th>
<th>Nomenclature</th>
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<th>Pol IV</th>
<th>Pol V</th>
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<td>+</td>
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<td>+</td>
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**Culture Conditions, Media, and Titering Assays**

Strains were cultured in 5.0mL LB Broth, Lennox (Difco-BD) and incubated at 37°C with aeration in a TC-7 test tube roller (New Brunswick Scientific), unless otherwise specified. Experiments were initiated from overnight cultures inoculated from frozen LB-glycerol stocks. Viable counts were determined by serial dilution of cells periodically removed from cultures, and plating on selective medium containing the appropriate antibiotics: Nal, nalidixic acid (20µg/mL); Str, streptomycin (25µg/mL); Spc, spectinomycin (100µg/mL); Kan, kanamycin (50µg/mL); Cam, chloramphenicol (30µg/mL); and Rif, rifampicin (100µg/mL). This method of titering is accurate within +/- 3-fold (Kraigslay and Finkel 2009), and has a limit of detection of 1000 colony forming units (CFU)/mL in this study.

**Batch Culture Long-term Competition Assays**

Two types of batch culture competitions, distinguished by their initial cell densities, were performed: Type 1, where both strains are mixed at a low initial density (~10<sup>6</sup> CFU/mL) and co-outgrown, and Type 2, where high-density stationary phase cultures are mixed (~10<sup>9</sup> CFU/mL). In Type 1 experiments, competitions were initiated by inoculating 5µL of each competing strain
(1:1000 dilution, vol:vol) into the same 5.0mL LB culture. In Type 2 experiments, competitions were initiated by combining 2.5mL of overnight stationary phase cultures of each strain (1:1 mix, vol:vol). Viable counts were determined as described above using the appropriate combinations of antibiotics (Kan/Cam for Pol II\textsuperscript{+}-only, Spc/Cam for Pol IV\textsuperscript{+}-only, and Spc/Kan for Pol V\textsuperscript{+}-only). At the conclusion of each 14-day competition, strains showing a 10-fold greater relative population density were scored as winners.

**Serial Passage Aging Regimen**

To observe changes in relative fitness following repeated outgrowth, strains were serially passaged in 5.0mL LB cultures. Independent cultures of each strain were incubated for 24 hours, sampled and diluted 1:1000 (vol:vol) into fresh medium. Cultures were passaged daily for 5 days and frozen LB-glycerol stocks were prepared following each passage.

**Selection of GASP Mutants and GASP Competitions**

To select for GASP mutants, strains were inoculated into independent 5.0mL LB cultures, incubated for 10 days, and 150\(\mu\)L was frozen as LB glycerol stocks. To initiate GASP competitions, an overnight culture of each aged population was grown and introduced as a minority at a 1:1000 (vol/vol) dilution into a 5.0mL culture of an unaged population of wild type cells (ZK1142) or polymerase mutant strains (ZAMBRANO et al. 1993). Population densities for each strain were determined by titering, as described above.

**Chemostat Competitions**
To assess the relative fitness of polymerase-deficient strains under conditions promoting rapid growth, competitions were performed under continuous culture conditions in chemostats (Chao and Cox 1983; Harder and Kuenen 1977). For each competition, 250µL (1:3000, vol:vol) of each double-mutant strain (Pol II⁺, IV⁺ and V⁺) was inoculated into 750mL LB in a BioFlo 110 bioreactor (New Brunswick Scientific). The chemostat was run for up to 2 hours under batch conditions to obtain the desired density before initiating flow of fresh medium into the growth chamber. Average dilution rates varied from 1 to 4 volumes per hour and chemostats were run between ~6 to 10 hours. The chemostat culture was regularly sampled to monitor optical density and determine viable counts.

**Mutation Frequency Assay**

The frequency of spontaneous rifampicin resistance was determined in wild type and all seven polymerase-deficient strains. For each strain, 159 independent 5.0mL overnight cultures were grown and 100µL of each was spread onto plates containing rifampicin. Total cell counts were determined by plating an appropriate dilution of each culture on LB agar. The number of RifR colonies was determined after 48h of incubation at 37°C. The frequency of spontaneous rifampicin resistance was calculated by dividing the number of RifR CFU/mL by the total CFU/mL. The distributions of observed RifR frequencies were compared using the two-sample Kolmogorov-Smirnov (K-S) Test (p < 0.05)(http://www.physics.csbsju.edu/stats/KS-test.html). This nonparametric test can be applied to compare the cumulative distribution function of two empirical distributions of continuous data to determine the likelihood both sets were obtained from the same distribution. Importantly, this test statistic has the advantage that it is not contingent upon assumptions of normalcy in the data and is capable of assessing the cumulative
distribution of mutation frequencies rather than relying strictly upon mean or median values
which might not accurately reflect alterations across the cumulative distribution of the data.
Differences in average RifR frequencies were assessed using Standard Error of the Mean (SEM).

RifR Mutant Sequencing

The sequences of RifR mutants were determined using cells obtained from the mutation
frequency assay described above. The RifR colony nearest the center of each plate was
resuspended in 20µL LB. 2µL was used as template for PCR amplification of the rpoB gene
using Primer 1 (5’-AATGTCAAATCCGTGGCGTG-3’) and Primer 2 (5’-
TTCACCCGATACATCTCGTC-3’), with the remaining sample frozen in LB-glycerol.
Amplified products were sequenced at High-Throughput Sequencing Solutions (Seattle, WA)
using Primer 1 or Primer 3 (5’-CGTCGTATCCGTCCGTGG-3’) specific for Cluster I and
Cluster II, respectively (GARIBYAN et al. 2003).

Quantitative RT-PCR

Real-time RT-PCR was used to determine the expression patterns of each alternative
polymerase gene, as well as induction of the SOS response. LB cultures inoculated with a 1:1000
dilution of an overnight wild type population were incubated at 37°C and periodically sampled
for RNA extraction. Samples were treated with RNAlater (Qiagen) and total RNA was isolated
using the RNeasy Mini Kit (Qiagen). qRT-PCR reactions with 100ng template RNA were
performed using the One-step RT-PCR Kit (Qiagen) with SYBR Green (Molecular Probes) and
amplified on an Opticon-2 Real-time PCR Cycler (MJ Research). Primers used for amplification
are provided in Table S4. Relative transcript abundance and changes in gene expression were
determined using the $2^{-\Delta C_{\text{t}}}$ method (Livak and Schmittgen 2001). In control experiments with artificial SOS induction, mitomycin C (Sigma) was added to 1 $\mu$g/ml final concentration after 2 hours of incubation, at an OD$_{600}$ of ~0.1, and RNA was sampled hourly.

Supporting Information

**Figure S1: Outgrowth of Polymerase-deficient Mutant Strains**

Cell densities of the wild type and all combinations of mutant strains are plotted for 24 hours of growth in LB. Timepoints were taken every 45 minutes for the first 12 hours and every 90 minutes during the second 12 hours. All strains exhibited indistinguishable growth curves; representative data are shown. Black, wild type; light red, Pol II$^-$; light green, Pol IV$^-$; light blue, Pol V$^-$; dark blue, Pol V$^+$-only; dark green, Pol IV$^+$-only; dark red, Pol II$^+$-only; gray, triple mutant.

**Table S1. Rif$^R$ Single-nucleotide Polymorphisms Identified**

All missense mutations in *rpoB* conferring rifampicin resistance (Rif$^R$) identified in this study are listed, along with the number observed within each strain background. Asterisks denote missense mutations conferring rifampicin resistance that to our knowledge have not previously been reported.

**Table S2. Rif$^R$ Deletions and Amplifications Identified**

All unique deletions and amplifications in *rpoB* conferring rifampicin resistance (Rif$^R$) identified in this study are listed. Some deletions were identified more than once within a given strain. The codons affected are provided.

**Table S3. Strain-specific Mutation Spectrum**

The absolute number of mutations identified in each class of mutation, as well as the percent of overall mutations they represent, within each strain is provided.

**Table S4. qRT-PCR Primers used in this study**

Acknowledgements

We thank Phuong Pham, Roger Woodgate, and members of the Finkel Lab for helpful discussions and Ken Nealson and Andrea Cheung for assistance with chemostat experiments. This work was supported in part by a grant from the U.S. Air Force Office of Scientific Research (FA-9550-06-1-0292) and an NSF CAREER Award (MCB0237975) to S.E.F. and NIH grants GM21422 and ES012259 to M.F.G. C.H.C. received support from the NIH Cellular, Biochemical and Molecular Sciences Training Program (T32GM67587).
Figure Legends

Figure 1. Type 1 and Type 2 Competition Experiments Between Double-Mutant Strains.
Representative Type 1 low initial cell density (A-C) and Type 2 high initial cell density (E-G) competitions between unaged polymerase-deficient strains: red lines, Pol II⁻-only; green lines, Pol IV⁺⁻only; blue lines, Pol V⁺⁻only. Color-coded Roman numerals in each panel refer to the polymerases expressed in each pair of mutant strain competitions. Three representative competitions are shown where squares, circles, and triangles indicate competition pairs. Competition data, summarized in (D) & (H), reflect the results of 9 competition experiments for each of the three pairings of mutant strains. The “>” sign indicates that the strain listed on the left was the “winner;” the “<” sign on the right indicates that the strain listed on the right was the “winner.” One strain outcompeting the other is defined by a >10-fold difference in cell yield on day 14. When final yields are within ten-fold, no winners were declared as reflected by the “=” sign. Asterisks indicate that cell titers were below the limit of detection (<1000 CFU/ml.)

Figure 2. GASP Competitions Between Wild Type and Double-Mutant Strains. Polymerase double-mutant strains were aged for 10 days and competed to determine their GASP phenotypes against unaged wild type strains (A-C) or each unaged polymerase mutant strain (D-I), in all possible combinations. Filled symbols correspond to strains aged for 10 days; open symbols correspond to unaged strains. Strains are indicated by line color: wild type, black; Pol II⁻-only, red; Pol IV⁺⁻only, green; Pol V⁺⁻only, blue. Unaged wild type cells were competed against 10-day-old (A) Pol II⁻-only, (B) Pol IV⁺⁻only, or (C) Pol V⁺⁻only. Aged Pol II⁻-only strains were competed against unaged (D) Pol IV⁺⁻only or (G) Pol V⁺⁻only; aged Pol IV⁺⁻only strains were competed against unaged (E) Pol II⁻⁻only or (H) Pol V⁺⁻only; and aged Pol V⁺⁻only strains were competed against unaged (F) Pol II⁻⁻only or (I) Pol IV⁺⁻only. Three representative competitions are shown for each pair where squares, circles, and triangles indicate competition pairs. Asterisks indicate that titers were below the limit of detection (<1000 CFU/ml.) Color-coded Roman numerals in each panel refer to the polymerases expressed in each pair of mutant strain competitions.

Figure 3. Increased Relative Fitness of Pol II⁺⁻only Mutants Following Serial Passage. Populations of unaged Pol IV⁺⁻only (green lines, A-E) or unaged Pol V⁺⁻only (blue lines, F-J) were each competed against the Pol II⁺⁻only strain (red lines) after one to five serial passages. Panels A& F show competition between the Pol II⁺⁻only strain with no additional passage; (B & G) show competitions after one additional passage of Pol II⁺⁻only; (C & H) two additional passages of Pol II⁺⁻only; (D & I) three additional passages of Pol II⁺⁻only; and (E & J) five additional passages of Pol II⁺⁻only. The average relative log₁₀ ratio of final cell densities between Pol II⁺⁻only versus Pol IV⁺⁻only (green bars) or Pol V⁺⁻only (blue bars) for all six conditions is plotted in (K).

Figure 4. Relative Fitness During Chemostat Competitions. The relative fitness of Pol II⁺⁻only compared to Pol IV⁺⁻only (in green) and Pol V⁺⁻only (in blue) during continuous culture competitions is shown for chemostats run with different dilution rates (volumes per hour).

Figure 5: Strain-specific Rif⁺ Mutation Frequency and Spectrum.
The frequency of spontaneous rifampicin resistance was determined for 159 independent replicates of the wild type and each polymerase mutant strain. Mutation frequencies are presented in ascending order. (A) Mutation frequencies for the wild type (black), all three double-mutant strains (Pol II\(^{+}\)-only, red; Pol IV\(^{+}\)-only, green; Pol V\(^{+}\)-only, blue), and the triple mutant (gray). (B) Mutation frequencies for the wild type (black), all three single-mutant strains (Pol II\(^{-}\), red; Pol IV\(^{-}\), green; Pol V\(^{-}\), blue), and the triple mutant (gray). (C) Average Rif\(^{R}\) frequencies for the wild type, all three single-mutant strains, all three single double mutant strains, and the triple mutant. Error bars denote +/- Standard Error of the Mean. (D) For all eight strains, each class of mutation is presented as a percentage of all mutations observed. Detailed mutation data is presented in Tables S1-3.

**Figure 6: Alternative Polymerase Transcript Abundance Changes Over the Cell Cycle.** mRNA transcript abundance, in the absence of exogenous SOS inducers, was determined by qRT-PCR. (A) Transcript abundance for each gene relative to its concentration at 2 hours of incubation. Genes are identified as: *polB*, red, open circles; *dinB*, green open triangles; *umuC*, blue, open squares; *umuD*, blue, closed squares; *dnaE*, black diamonds; *sulA*, purple, filled circles; *lexA*, orange, filled triangles. (B) The proportion of polymerase transcripts over time, expressed as a percentage of total transcript abundance, is shown for four representative transcripts: Pol III, *dnaE*, black diamonds; Pol IV, *dinB*, green triangles; Pol II, *polB*, red circles; Pol V, *umuC*, blue squares.
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Figure 1

A - C: Comparison of log$_{10}$ CFU/mL over 14 days for Type 1: Low Initial Cell Density.

A: II vs IV
B: II vs V
C: IV vs V

D: Summary of statistical tests for Type 1:

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E - G: Comparison of log$_{10}$ CFU/mL over 14 days for Type 2: High Initial Cell Density.

E: II vs IV
F: II vs V
G: IV vs V

H: Summary of statistical tests for Type 2:

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<tr>
<td>Pol IV$^+$</td>
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Figure 2

A-C: Comparison of CFU/mL for different groups over a period of days.

D-G: Detailed comparisons between specific groups (II vs WT, II vs IV, II vs V).

H: Comparison of IV vs WT, IV vs II, IV vs V.

I: Comparison of V vs WT, V vs II, V vs IV.
Additional Passages

Figure 3
Figure 4

Average Dilution Rate (Volumes/Hour) vs. Avg. Pol II+ vs. Pol IV+ or Pol V+ Ratio
Distribution of RifR Frequencies

Double Mutants
- Wildtype
- Pol V+-only
- Pol IV+-only
- Pol II+-only
- / - / -

Single Mutants
- Wildtype
- Pol II-
- Pol IV-
- Pol V-
- / - / -

Replicate Number

$\log_{10}$ (RifR Frequency)

A

Average RifR Frequency

C

Strain-specific Mutation Percentages

D

Strain
- WT
- II-
- IV-
- V-
- V+-only
- IV+-only
- II+-only
- -/-/

Distribution of RifR Frequencies

Single Mutants

A

Average RifR Frequency

C

Strain-specific Mutation Percentages

D

Percent of Mutations

Mutation Class
- GC $\rightarrow$ AT
- GC $\rightarrow$ TA
- GC $\rightarrow$ CG
- AT $\rightarrow$ GC
- AT $\rightarrow$ TA
- AT $\rightarrow$ CG
- Deletions
- Insertions

Figure 5
Figure 6

A

Transcript abundance relative to Hour 2

polB  
dinB  
umuC  
umuD  
umuD  
dnaE  
sulA  
lexA

B

Proportion of transcript abundance

2 4 6 8 10 12 14

0%  20%  40%  60%  80%

2 4 6 8 10 12 14

0%  20%  40%  60%  80%