SOS-induced DNA polymerases enhance long-term survival and evolutionary fitness

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Escherichia coli **encodes three SOS-induced DNA polymerases: pol II, pol IV, and pol V. We show here that each of these polymerases confers a competitive fitness advantage during the stationary phase of the bacterial life cycle, in the absence of external DNA-damaging agents known to induce the SOS response. When grown individually, wild-type and SOS pol mutants exhibit indistinguishable temporal growth and death patterns. In contrast, when grown in competition with wild-type *E. coli*, mutants lacking one or more SOS polymerase suffer a severe reduction in fitness. These mutants also fail to express the ''growth advantage in stationary phase'' phenotype as do wild-type strains, instead expressing two additional new types of ''growth advantage in stationary phase'' phenotype. These polymerases contribute to survival by providing essential functions to ensure replication of the chromosome and by generating genetic diversity.**

GASP | genetic diversity | competitive fitness | stationary phase survival | SOS DNA polymerases

Of the five DNA polymerases present in *Escherichia coli*, three are induced as part of the SOS regulon in response to DNA damage (1). Pol V, encoded by *umuDC* (2–4), and pol IV, encoded by *dinB* (5), are members of the recently named Y-family of error-prone polymerases (6), whereas B-family pol II, encoded by *polB* (7–11), copies DNA with high fidelity (12). Although we generally think of these enzymes as being either error-free or error-prone polymerases, under certain conditions, an error-prone polymerase can catalyze an error-free reaction and *vice versa* (1). SOS pols take part in diverse biochemical pathways in dividing and stationary-phase cells. In dividing cells, pol V is responsible for generating base substitution mutations targeted at DNA damage sites (13, 14). Pol II is instrumental in rescuing stalled replication forks on damaged DNA (15, 16), but in contrast to error-prone pol V, the pol II-catalyzed replication-restart process occurs with no measurable increase in mutational load (15). Indeed, pols II and V behave as ''flip sides of a coin''—pol II acting early to help catalyze ''error-free'' replication–restart and pol V coming into play only later to perform translesion synthesis at persisting DNA damage sites (13, 15, 17, 18). Pol IV may be primarily involved in generating simple ''nontargeted'' frameshift mutations (19, 20), perhaps while helping to rescue replication forks that become stalled on undamaged DNA (21). Pol IV is also involved in copying bulky template adducts (22, 23). In nondividing cells, pols IV and II play a central balancing role during adaptive mutation, a process in which nonproliferating microbial populations accumulate mutations when placed under nonlethal selective pressure (24). Pol IV is responsible for making most of the *lacZ* adaptive frameshift mutations occurring on a plasmid (25, 26), with pol II acting to moderate the level of mutation (27).

A stationary-phase-specific process, the expression of the ''growth advantage in stationary phase'' (GASP) phenotype, depends on the appearance of new mutations in the population (28–30). The new mutations confer a competitive advantage to cells, allowing them to take over the population. The appearance of the GASP phenotype is a key metric characterizing the dynamic state of cell populations (31, 32) during stationary-phase incubation. In this article, we examine the growth, survival, and ability to express the GASP phenotype of all combinations of SOS polymerase mutants in competition with themselves, with each other, and with wild-type cells in the absence of exogenous DNA-damaging agents during stationary phase. Bacteria existing in natural environments experience cycles of feast and famine, but much more time is likely to be spent in a famine mode (33). The growth of bacteria under conditions of long-term stationary phase incubation serves as an excellent model for these long periods of nutrient stress.

Materials and Methods

Culture Conditions, Media, and Titering Assays. All strains were cultured in LB broth and incubated at 37°C with aeration in a test tube roller. Viable counts were determined by serial dilution of cells removed periodically from the culture, followed by plating on LB agar containing the appropriate antibiotics used at the following concentrations: nalidixic acid (20 μg/ml), streptomycin (25 μg/ml), spectinomycin (100 μg/ml), kanamycin (50 μg/ml), and chloramphenicol (30 μg/ml). To measure long-term survival of individual strains, 5-ml LB cultures were inoculated 1:1,000 (vol/vol) from fresh overnight cultures started directly from frozen LB-glycerol stocks. Viable counts were determined directly from cultures incubated for up to several weeks. To determine relative fitness, wild-type and polymerase mutant strains carrying different antibiotic markers were coinoculated 1:1,000 as described above. Individual subpopulation titers were determined after periodic sampling, serial dilution, and plating on media containing appropriate antibiotics. GASP experiments were performed as described (28, 31) by using aged cells which were incubated continuously for 10 days at 37°C with aeration without the addition of nutrients.

Bacterial Strains Used. All experiments were performed with strains derived from *E. coli* K-12 strain ZK126 (W3110 Δ*lacU169 tna-2*), including the nalidixic acid- and streptomycin-resistant wild-type strains ZK1142 and ZK1143, respectively (28). DNA polymerase null mutants were constructed by bacteriophage P1 transduction into ZK126 by using the following donor strains: for pol II$^-$, SH2101 *polB*::Spc (34); for pol IV$^-$, RW626 *dinB*::Kan (Kan-insertion mutation derived from strain EYT2; H. Ohmori, personal communication); and for pol V$^-$, RW82 *umuDC*::Cam (both RW626 and RW82 are gifts of R. Woodgate, National Institutes of Health, Bethesda, MD). Mutations or genetic elements conferring antibiotic resistance are effectively neutral in the absence of drug selection (28, 31). Possible polar effects of insertion mutations are of concern for the *dinB*::Kan strain. To address this possibility, another *dinB* null allele, carrying two in-frame stop codons (*dinB-STOP4*), was constructed. This strain (SF2219) also shows a fitness defect (data not shown). In addition, the pol IV$^-$ fitness reduction phenotype was comple-

Abbreviations: GASP, growth advantage in stationary phase; pol II, DNA polymerase II; pol IV, DNA polymerase IV; pol V, DNA polymerase V; RT, reverse transcription.

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mented by expression of wild-type pol IV protein from a plasmid (data not shown).

Reverse Transcription Assays. Reverse transcription–PCR (RT-PCR) reactions (OneStep RT-PCR Kit, Qiagen, Chatsworth, CA) were performed according to the manufacturer's instructions on whole-cell RNA (RNeasy, Qiagen) isolated from wild-type and pol mutant cells grown for 1–5 days. Oligomer probes were used to amplify mRNA for the *polB*, *dinB*, *umuD*, and *recA* genes, all induced during the SOS response, or the stationary phase-specific *dps* (35) and *sbmC* (36) genes. The following primer pairs were used to amplify each gene: *polB*, Top: GCATTAACGATAGTGCCATTG, Bottom: CCGCAAGGGACAGAAGTCTC; *dinB*, Top: CATTGAACCGTTGTCACTGG, Bottom: GTAATCACAAACTGGCCGTTG; *umuD*, Top: GTGTGGCTTTCCTTCACCG, Bottom: CTTCACTACTGATGGTAATG; *recA*, Top: GCGTCACAGATTTCCAGTGC, Bottom: GGTAAAGGCTCCATCATGC; *sbmC*, Top: GCAAAATCATCACCGACTACAC, Bottom: CGAGATTAAGCAGGAAGAG; *dps*, Top: GTTGATAACTTGAGTGGTCC, Bottom: GTAGAGTTGCTGAATCGCCAG.

Results

When cultured under standard laboratory conditions in rich medium, null mutations of each of the three SOS pols have no observable effect on cellular growth (Fig. 1*A*). Individually cultured pol$^-$ cells show the same cell yields after overnight growth and the same stationary-phase survival patterns as the wild-type. In fact, mutants of pol II, pol IV, and pol V can survive at least 2 months of stationary-phase incubation, without the addition of nutrients, at cell densities similar to the wild-type (data not shown). However, in an environment where wild-type and mutant strains are cocultured and must compete for nutrients, the situation changes dramatically. Each pol$^-$ strain displays a significant reduction in relative fitness leading to its eventual extinction from the population when cocultured with the isogenic wild-type parent (Fig. 1*B*). Mutant cells deficient in pol II, pol IV, or pol V cannot survive more than 10 days of coculture with wild-type cells. Each mutant strain can survive for up to 5 days but then begins to disappear, decreasing at rates of up to an order of magnitude per day. After 10–14 days of incubation, no pol$^-$ mutant cells remain in the culture. Double mutants of each pairwise combination of pol$^-$ alleles, as well as the pol II$^-$, pol IV$^-$, and pol V$^-$ triple mutant, show fitness losses during coculture more quickly than the single mutants (data not shown).

We have verified that SOS polymerase genes are transcribed during stationary phase in the absence of exogenous DNA damage (Fig. 2). RT-PCR products corresponding to transcripts of all three SOS polymerase genes and *recA* have been obtained from cells incubated for 1–5 days. In addition, we have observed RT-PCR products of transcripts corresponding to the stationary-phase-specific gene *dps* (35), encoding a highly abundant, nonspecific DNA-binding protein, and *sbmC*, which encodes a gene regulating susceptibility to the peptide antibiotic microcin B17. *sbmC* is expressed both during stationary phase and upon SOS induction (36). Null mutants of each DNA polymerase strain do not yield an RT-PCR product (Fig. 2*A*).

Each pol$^-$ mutant shows a competitive disadvantage when cocultured with the wild-type parent (Fig. 1*B*), yet the mutants show similar relative fitness when competing against each other. In this case, we do not mean to imply that the two competing pol$^-$ (null mutant) populations remain at high density for the entire incubation period. Instead, we observe that when many initially identical cocultures are prepared, roughly half of the time, one pol$^-$ strain dominates and half of the time the other mutant wins. This "flip-flop" in survival suggests that each pol$^-$ mutant accumulates either deleterious or possibly advantageous

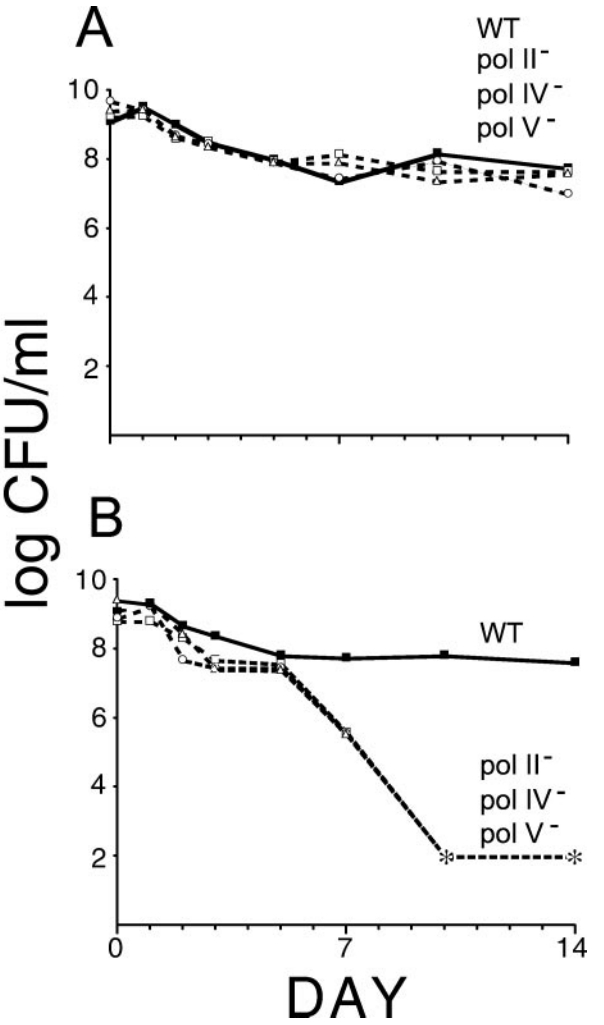


Fig. 1. Long-term survival patterns of wild-type and polymerase mutant cells. (*A*) Composite of survival patterns when cells are grown alone in batch culture. (*B*) Composite of survival patterns of each pol$^-$ strain when grown in competition with wild-type cells. Representative growth curves are shown. Solid line with filled boxes, wild-type; dashed lines are mutant strains: open squares, pol II$^-$; open circles, pol IV$^-$; open triangles, pol V$^-$ mutants. CFU/ml, colony-forming units per milliliter. Asterisks correspond to cell titers below the limit of detection of 100 CFU/ml.

mutations similarly, resulting in similar overall fitness profiles. We show a representative experiment in which two different polymerase null mutant strains, in this case mutants of pol IV and pol V, are cocultured and incubated under long-term stationary-phase conditions (Fig. 3). In each trial of the experiment, neither strain completely outcompetes the other. More pol V$^-$ cells exist relative to pol IV$^-$ cells at the conclusion of one trial (Fig. 3*A*), but more pol IV$^-$ cells than pol V$^-$ at the end of the second trial (Fig. 3*B*). In the third trial, although pol IV$^-$ cells outnumber pol V$^-$ cells at the end of the experiment, the pol V$^-$ cells are increasing in relative numbers (Fig. 3*C*). Several iterations of these experiments with all three possible pairs of competing strains (pol II$^-$ vs. pol IV$^-$, pol II$^-$ vs. pol V$^-$, and pol IV$^-$ vs. pol V$^-$) showed no clear winners or losers. None of the pol$^-$ strains displayed an overall competitive advantage or disadvantage versus other pol$^-$ strains, whereas their lack of competitive fitness was obvious when growing in the presence of wild-type cells (Fig. 1*B*).

Long thought of as a period of stasis, the stationary phase of the bacterial life cycle during batch culture incubation is now

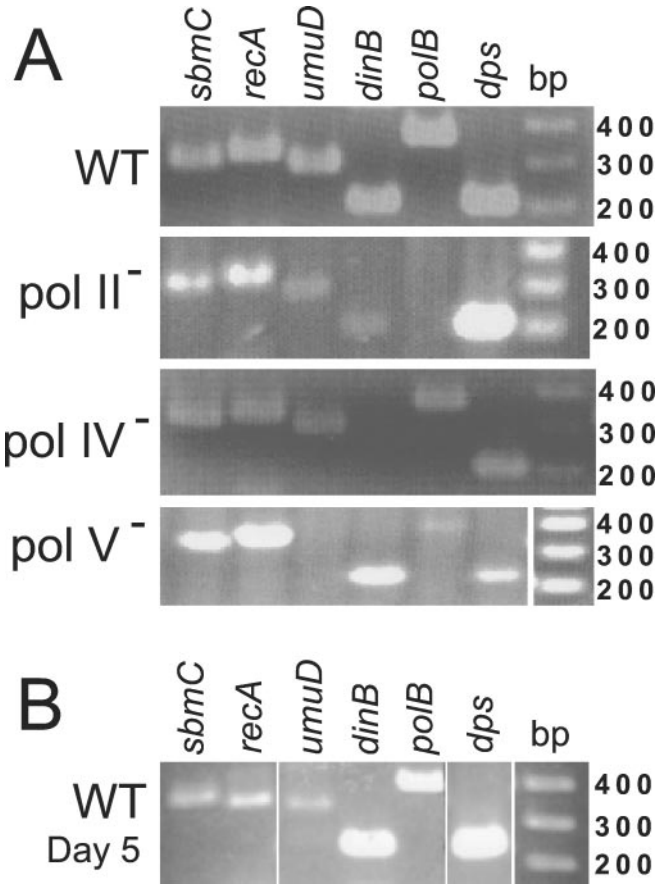


Fig. 2. Transcription of the SOS-induced DNA polymerases. RT-PCR assays were performed on wild-type (WT), *polB* (pol II$^-$), *dinB* (pol IV$^-$), or *umuDC* (pol V$^-$) cells harvested from 1-day-old cultures (*A*) or 5-day-old wild-type cultures (*B*). Gene names are indicated above each lane. The expected sizes of RT-PCR products are: *sbmC*, 311 bp; *recA*, 328 bp; *umuD*, 298 bp; *dinB 206 bp,* polB, 367 bp; *dps*, 209 bp.

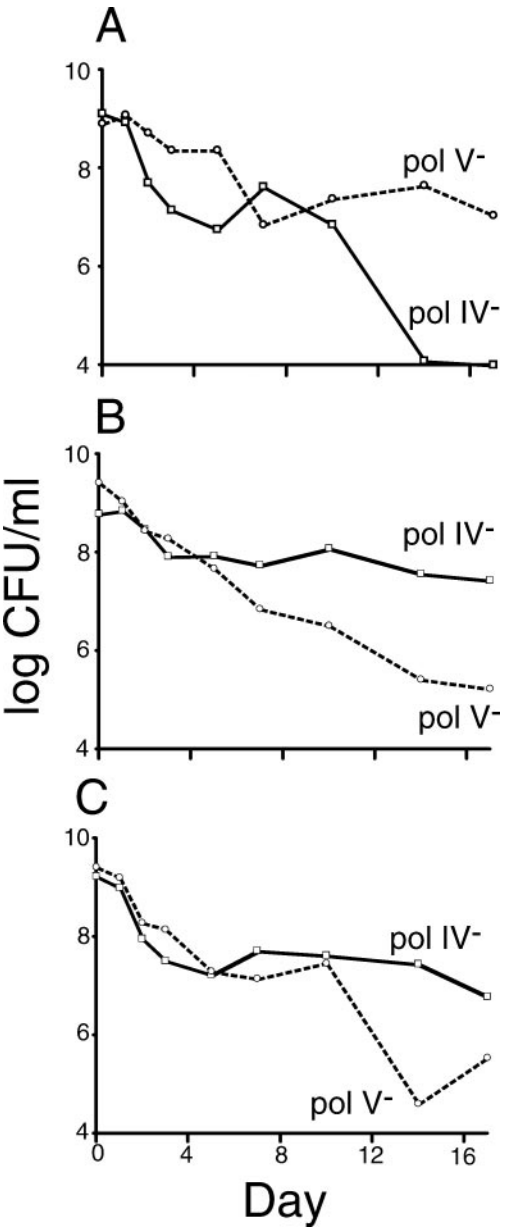


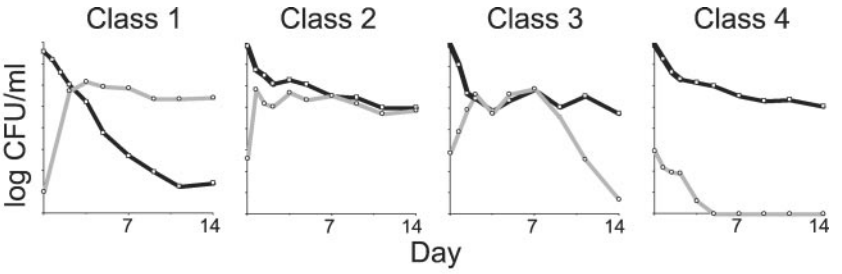
Fig. 3. Representative competitions between polymerase-deficient strains. Solid lines with open squares, pol IV$^-$; dashed lines with open circles, pol V$^-$. CFU/ml, colony forming units per milliliter.

known to be a highly dynamic period during which many genetic systems are induced that ensure the long-term survival of the population (30, 32). As incubation continues in the absence of new nutrients, further cellular changes occur eventually leading to the death of more than 99% of cells. It is from the survivors of this "death phase" that another change soon becomes apparent. Among this population emerge cells expressing the Growth Advantage in Stationary Phase, or GASP, phenotype (28, 29). GASP results from the appearance and selection of advantageous mutations that confer a competitive fitness advantage on a subset of cells within the population during stationary phase.

Previous data (Fig. 1) demonstrate that each SOS polymerase confers an important advantage for survival on a short-term basis. We now address whether these polymerases also play a role in the long-term survival of bacteria in stationary phase during nutrient stress. In other words, do SOS polymerase mutants exhibit the GASP phenotype as do their wild-type parents?

In a typical GASP competition experiment, cells from a 10-day-old wild-type culture are inoculated as a numerical minority (1:1,000 vol/vol) into a fresh (1-day-old) culture. Both subpopulations are monitored for the next few weeks, and their relative proportion in the population is determined. When wild-type cells are aged for 10 days, they invariably display the GASP phenotype, completely outcompeting the parental nonaged strain after 10–12 days of coculture (Fig. 4, class 1). The results are dramatically different when the GASP analysis is performed by using SOS polymerase mutants (Fig. 4). Individual cultures of wild-type or pol$^-$ strains were cultured for 10 days in LB. After 10 days, a 5-μl sample of each culture was inoculated directly into 1-day-old 5-ml cultures of the wild-type parent. Wild-type and pol$^-$ mutant strains carry unique antibiotic resistance markers allowing each to be distinguished. Unlike wild-type cells that display a strong GASP phenotype (class 1) after 10 days of incubation, in virtually every case, the pol$^-$ mutant strains display up to four different phenotypes or "flavors" of GASP.

In addition to the class 1 competition, where aged cells completely outcompete nonaged cells, we also observed competition in which aged cells were able to increase their relative frequency in the population displaying equal fitness to the nonaged wild-type parent (class 2). A third type of competition (class 3) is observed where the aged cells attempt, but fail, to



GASP Class Frequency (%)

Strain	Class 1	Class 2	Class 3	Class 4
WT	100	0	0	0
pol II$^-$	21	65	7	7
pol IV$^-$	43	36	0	21
pol V$^-$	33	11	17	39

Fig. 4. GASP competition phenotypes of wild-type and polymerase mutant strains during coculture. Competition phenotypes are defined as: class 1, strong GASP phenotype; class 2, weaker GASP phenotype; class 3, abortive expression of GASP; class 4, no expression of the GASP phenotype. Representative examples of each class are shown. Black lines represent titers of unaged populations; gray lines represent titers of cells obtained from 10-day-old aged cultures. The frequency, in percentage, of expression of each class of GASP competition phenotype for wild-type and mutant strains is shown. $n = 14$ for wild-type (WT), pol II$^-$, and pol IV$^-$ strains; $n = 18$ for pol V$^-$ strains. CFU/ml, colony forming units per milliliter.

outcompete the wild-type. These pol mutants are less fit, because, after initially expressing a growth advantage, increasing their relative frequency in the population for a short time, they are completely displaced by nonaged parental cells. A last competition class is observed (class 4) where aged mutant strains are completely unable to compete with the nonaged wild-type parent. Previous studies of the ability of wild-type cells to express the GASP phenotype (28–32) revealed only two competition classes (equivalent to classes 1 and 4 described here.) This study identifies two new classes of GASP (classes 2 and 3) in which the potential competitive advantages gained by the acquisition of new alleles are countered by the competitive fitness loads of carrying mutant pol alleles.

Because of the disparate fidelity properties of the three SOS polymerases, it might be expected that the acquisition of mutations conferring a competitive advantage during stationary-phase growth may differ depending on the pol mutant allele. It seems that pol V may play a more significant role in the acquisition of the GASP phenotype, whereas pol II and IV mutants acquire a GASP phenotype about 86 and 79% of the time, respectively (including both class 1 and 2 competitions), pol V mutant strains GASP only 44% of the time. Although the pol V mutants express the GASP phenotype less frequently than the other two mutant strains, when the pol V$^-$ cells do express a competitive advantage, it is usually of the stronger phenotypic class (class 1). In other words, aged pol V$^-$ strains are very good competitors almost as frequently as they are very poor competitors (Fig. 4).

Although SOS polymerase-deficient strains do not always express the GASP phenotype when competing with their wild-type parent, they do express GASP when competing with other pol$^-$ strains. In almost every case, aged 10-day-old pol$^-$ cells, when inoculated as a minority into a young culture, express the GASP phenotype, outcompeting the nonaged (1-day-old) mutant strains in the majority (data not shown). The one exception was for GASP competitions between aged pol V$^-$ mutant strains and nonaged pol IV$^-$ strains. In these experiments, pol V mutants failed to express the GASP phenotype in two of three trials (data not shown). The interesting question raised by these results is whether mutations that appear during long-term incubation that do not confer the GASP phenotype are simply not the "right" kind of mutations to allow expression of a competitive advantage or whether the abundance of any beneficial mutation is countered by the load of a reduction in replication efficiency caused by carrying a pol$^-$ phenotype.

Discussion

When looking only at large populations of isogenic cells growing in batch culture, the types of fitness defects reported here are impossible to detect because the cells are indistinguishable: they all carry the same genetic markers. If an individual cell suffers a deleterious mutation, it is replaced by the progeny of viable siblings. Although growth rates seem to be normal and populations seem to be maintained during long-term incubation, these cultures are actually highly dynamic. GASP mutants with increased fitness appear in "waves" that can sweep through the culture as it ages (28, 29, 31, 32). Cells that are hobbled in their ability to grow, even to a relatively modest degree, will over time be out-competed by more fit cells. When the types of DNA lesions that induce the SOS polymerases are encountered, replication is impeded, causing a loss in competitive fitness. Each SOS polymerase may make its own specialized contribution to maintaining fitness and the generation of genetic diversity by filling in for a temporarily indisposed replicative pol III core polymerase during error-free replication-fork restart (pol II), putative error-prone replication fork rescue (pol IV), or error-prone translesion synthesis (primarily pol V.) Pols II and V also contribute to error-prone and error-free translesion synthesis depending on the specific identity of the lesion encountered (1, 22).

The relative fitness of any SOS polymerase mutant growing under the conditions of a GASP competition experiment reflects a balance between opposing selective forces: the appearance of positively selected advantageous mutations versus the negative effects of reduced replication efficiency. The competition data shown in Fig. 1*B* reflect the fitness load placed on each of the pol

mutant strains in a standard competition experiment. These fitness reductions may reflect the inability of these cells to replicate efficiently during long-term stationary-phase incubation. However, we do not believe that the reduced fitness phenotype during GASP competition is wholly attributable to reductions in replication efficiency. The observation that all three pol mutant strains are able to express a class 1 GASP competition phenotype (Fig. 4) suggests that the role of reduction in replication can be outweighed by a cell's ability to generate and maintain new advantageous alleles. Yet, the contribution of a loss in replication efficiency to this reduced fitness of SOS polymerase mutants remains an open question.

The observation that pol V mutants show the greatest reduction in class 1 GASP competitions suggests that a subset of mutations, attributable especially to pol V, may provide the mutational "raw material" on which natural selection acts during the evolution of bacterial populations, in a manner superficially similar to the increased fitness accompanying the absence of MutSLH mismatch repair system (37–40). A key distinction to be drawn between the kinds of mutational events associated with the loss of MutSLH mismatch repair and those associated with the loss of SOS polymerases is that replication persists, under most conditions, whether or not the mismatch repair system is functioning, allowing the propagation of missense mutations. When the types of DNA lesions that induce the repair polymerases are encountered, replication often halts, with dire consequences for the cell. The observation that the *polB*, *dinB*, and *umuDC* genes (along with known stationary-phase-specific genes, *dps* and *sbmC*) are transcribed during stationary phase in the absence of induction of the SOS system by exogenous DNA-damage agents suggests that the activities of these polymerases are essential for long-term survival. Given the observation that all three SOS pols can perform in both an error-prone or error-free manner, beyond the repair of DNA damage, these data support a model in which these alternative DNA polymerase enzymes are important for the generation of genetic diversity under normal physiological conditions.

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