Dynamics of Adaptation During Three Years of Evolution Under Long-Term Stationary Phase

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Abstract

Many bacterial species that cannot sporulate, such as the model bacterium *Escherichia coli*, can nevertheless survive for years, following exhaustion of external resources, in a state termed long-term stationary phase (LTSP). Here we describe the dynamics of *E. coli* adaptation during the first three years spent under LTSP. We show that during this time, *E. coli* continuously adapts genetically through the accumulation of mutations. For nonmutator clones, the majority of mutations accumulated appear to be adaptive under LTSP, reflected in an extremely convergent pattern of mutation accumulation. Despite the rapid and convergent manner in which populations adapt under LTSP, they continue to harbor extensive genetic variation. The dynamics of evolution of mutation rates under LTSP are particularly interesting. The emergence of mutators affects overall mutation accumulation rates as well as the mutational spectra and the ultimate spectrum of adaptive alleles acquired under LTSP. With time, mutators can evolve even higher mutation rates through the acquisition of additional mutation rate–enhancing mutations. Different mutator and nonmutator clones within a single population and time point can display extreme variation in their mutation rates, resulting in differences in both the dynamics of adaptation and their associated deleterious burdens. Despite these differences, clones that vary greatly in their mutation rates tend to coexist within their populations for many years, under LTSP.

Key words: experimental evolution, long-term stationary phase, rapid adaptation, mutators, convergent evolution.

Introduction

Evolutionary experiments are designed to study evolution as it occurs. Bacteria such as *Escherichia coli* are particularly useful for such experiments (reviewed in Barrick and Lenski 2013; Kassen 2014). Bacteria's great adaptability makes them an excellent model in which to study the dynamics of rapid adaptation. Short generation times enable investigators to rapidly observe bacterial evolution over many generations of growth. The ability to freeze bacterial populations, while maintaining their viability, allows one to "go back in time" and compare evolved populations to their ancestors. The relatively small size of bacterial genomes enables cheap and easy sequencing of many clones or population samples from different populations and time points of an experiment. This in turn provides greater resolution in determining the genetic changes associated with the observed evolutionary processes.

Most bacterial evolutionary experiments rely on setups that drive continuous or semicontinuous growth and which dilute out nonmultiplying cells (Barrick and Lenski 2013; Kassen 2014). This includes both experiments reliant on chemostats and on the serial dilution of evolving populations.

Yet, evolution likely often occurs within populations that are not continuously growing and in which the presence of temporarily nonmultiplying cells can greatly affect the dynamics of the evolutionary process (Brock 1971; Shoemaker and Lennon 2018). In order to better understand the dynamics of adaptation within such populations, it is useful to consider bacterial populations evolving under long-term stationary phase (LTSP). Bacterial species such as *E. coli* that cannot sporulate, can still survive for many decades within spent media (Zambrano et al. 1993; Finkel and Kolter 1999; Finkel 2006; Avrani et al. 2017; Chib et al. 2017). Upon inoculation into fresh media such bacteria will experience a short growth period, followed by a short stationary phase in which cell numbers remain constant. Shortly thereafter bacteria enter a rapid death phase. However, not all cells parish during this death phase. Instead, a small fraction of cells enter what is known as LTSP. In LTSP, population sizes reduce much more slowly, remaining fairly constant for months and even years. This allows for the maintenance of viability over many years under conditions of resource exhaustion. Mutation accumulation and fluctuations in genotype frequencies observed during the first few months under LTSP suggest that, at least for the few first months, cell replication occurs under LTSP, likely

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through the recycling of resources (Avrani et al. 2017; Chib et al. 2017).

In July 2015, our lab initiated evolutionary experiments designed to probe the dynamics of adaptation within resource-exhausted populations under LTSP (Avrani et al. 2017). Five independent LTSP populations were established, each by inoculating $\sim 5 \times 10^6$ E. coli K12 strain MG1655 cells/ml of Luria broth (LB), for a total volume of 400 ml, within 2-liter aerated flasks. The flasks were placed within an incubator set to 37 °C and are shaken at 225 rpm. Other than the addition of water to counteract evaporation, no new external resources were added to these flasks since. These ongoing LTSP populations are sampled periodically and frozen for later sequencing.

The five populations entered LTSP at about day 11 of the experiment. We have previously reported on our analyses of the dynamics of adaptation within these populations over the first four months (127 days) of the experiment (Avrani et al. 2017). Our results showed that over the first four months under resource exhaustion, LTSP populations genetically adapt through the rapid acquisition of mutations. We found the same genes and sometimes the same specific sites to be mutated across multiple independently evolving populations. Accumulated mutations were very strongly enriched for functional categories, which combined with the high levels of convergence observed suggest that mutation accumulation was governed by strong positive selection (Avrani et al. 2017).

Mutators, deficient in their DNA repair and suffering substantially higher mutation rates than their ancestral genotypes, were shown to frequently arise within lab-evolved microbial populations (e.g., Sniegowski et al. 1997; Giraud et al. 2001; Voordeckers et al. 2015). We observed the emergence of such mutatrors in three of our five LTSP populations (Avrani et al. 2017). Mutators were also observed at substantial frequencies within natural bacterial populations and among clinical antibiotic-resistant isolates (Gross and Siegel 1981; LeClerc et al. 1996; Mehta et al. 2019). Mutators likely increase in frequencies due to indirect selection in favor of linked adaptations (Wielgoss et al. 2013; Good and Desai 2016; Raynes et al. 2018). At the same time, there is also indirect selection against mutators due to linked deleterious mutations. Under conditions in which organisms are well adapted to their environment, more mutations will be deleterious than advantageous, leading to selection in favor of reduced mutation rates. Mutators will therefore likely rise to higher frequencies under conditions in which more adaptive mutations are available, or in other words, when a bacterial population is ill adapted to its environment (Wielgoss et al. 2013; Good and Desai 2016; Raynes et al. 2018).

When mutational input is limited, a new adaptive mutation will be unlikely to occur in the time it takes a previous adaptive mutation to fix. Under such limited mutational input, adaptation would thus be expected to occur through hard sweeps, at a rate limited by the availability of adaptive mutations. A pattern of clonal interference or soft sweeps is, in contrast, expected under conditions in which adaptive mutation supply is high, allowing several adaptive mutations to occur within a population, in close temporal proximity

(Fogle et al. 2008). We observed soft sweeps up to four months under LTSP, even when focusing only on nonmutators, suggesting that, even within nonmutators, mutational input was high enough as to not limit rapid adaptation through soft sweeps (Avrani et al. 2017).

In the current study, we expanded our analyses of the dynamics of adaptation under resource exhaustion to a much longer time frame of around three years (1,095 days). We show that LTSP populations continue to adapt through the highly convergent acquisition of mutations up to three years under resource exhaustion. For nonmutators, a majority of mutations appear to be adaptive. Adaptation seems to continue to not be limited by mutational input, as clonal interference is continuously observed. Our new data allow us to further examine the dynamics of mutator evolution. We find that the populations that originally evolved mutators by day 64 of our experiment continue to contain such mutators up to three years under LTSP. In contrast, the two populations that did not develop mutators early on, did not develop them later either. We further show that once mutators establish within a population, they can evolve into mutators with even higher mutation rates. Cells with very different mutation rates coexist at high frequencies for years within LTSP populations. Mutators suffering extremely high mutation rates suffer severe deleterious burdens. Despite such burdens, they can persist for years under LTSP, alongside cells with much lower mutation rates. Finally, we show that mutator phenotypes can affect not only the overall rates of mutation accumulation but also the spectrum of mutations and adaptations that occur.

Results and Discussion

LTSP Populations Maintain Constant Viability over the First three Years under Resource Exhaustion

The five LTSP populations established in July 2015 were initially sampled daily, then weekly, monthly, and later at longer intervals. Each sample was used to estimate viability within its population through the quantification of colony-forming units (CFUs) and was then archived by freezing at −80 °C. As can be seen in figure 1A, the populations initially grew to $\sim 10^{10}$ cells/ml, during the first day of the experiment. They then experienced a rapid death phase that slowed down at around day 11, which we consider to be the first day of LTSP. Populations continue to maintain fairly stable viable cell counts at least up to three years (1,095 days) under LTSP (fig. 1A).

Escherichia coli LTSP Populations Continuously Adapt in a Convergent Manner within Spent Media

We fully sequenced hundreds of clones, sampled from nine time points spanning the first three years spent under LTSP. At each population and time point sampled, approximately ten individual clones were sequenced (supplementary table S1, Supplementary Material online). The resulting short reads were aligned to the E. coli K12 MG1655 genome and mutations were called using the breseq pipeline (Deatherage and Barrick 2014). A full list of mutations found within all clones,
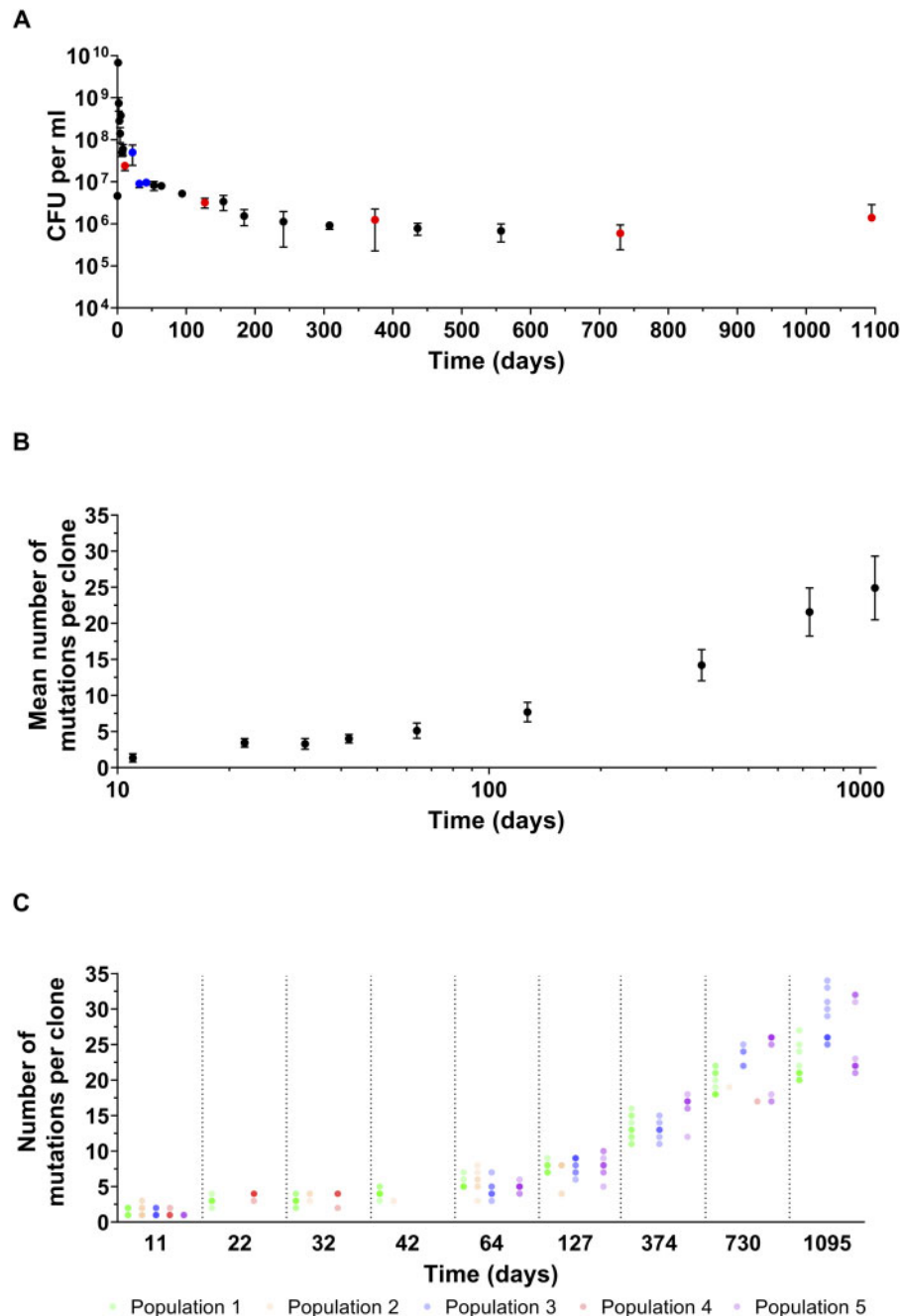
Fig. 1. Viable cell counts and mutation accumulation during the first three years under LTSP. (A) *Escherichia coli* populations maintain fairly constant viability levels up to 3 years under LTSP. Marks represent the mean number of cells/ml of LB, across the five populations as calculated through CFU quantification. Error bars represent the standard deviation (SD) around this mean. Time points at which approximately ten clones/ population were sequenced from all five populations are marked red. Time points at which approximately ten clones from populations 1, 2, and 4 were sequenced are marked blue. (B and C) Continuous mutation accumulation within nonmutator LTSP clones. (B) The mean number of mutations accumulated by LTSP clones increases in a continuous manner across the first three years under LTSP. Error bars represent the SD around the means. (C) Fairly consistent mutation accumulation across populations. Each mark represents an individual clone. Different mark shapes and colors indicate the population from which each clone was extracted. Clones with similar numbers of mutations are represented by overlaid dots, causing a darkening of the dot color.

including point mutations, indels, and IS element-mediated mutations, is given in supplementary table S2, Supplementary Material online.

Three of the five LTSP populations include mutator clones, which acquired a mutation within a mismatch repair gene. These clones accumulated a larger number of mutations, compared with nonmutator clones extracted from the same time point and population (supplementary table S3, Supplementary Material online, discussed at depth below).

First, we focused on nonmutator clones to examine the rates with which they accumulate mutations with time. As can be seen in figure 1B, nonmutator clones continue to

Table 1. Enrichment of Nonsynonymous Relative Synonymous Substitutions in Nonmutator Clones up to three Years under LTSP.

Time Point (days)	Number of Nonsynonymous Substitutions	Number of Synonymous Substitutions	dN/dS[a]	P value[b]
11	49	0	Cannot calculate	<0.0001
22	65	0	Cannot calculate	<0.0001
32	38	0	Cannot calculate	0.0006
42	29	1	8.9	0.0091
64	99	0	Cannot calculate	<0.0001
127	142	8	5.5	<0.0001
374	151	23	2	0.0014
730	258	36	2.2	<0.0001
1,095	393	48	2.5	<0.0001

[a]dN/dS (the ratio of the rates of nonsynonymous to synonymous mutations) was calculated as follows:

$$\frac{dN}{dS} = \frac{\frac{\text{Number of non-syn new mutations}}{\text{Number of non-syn sites}}}{\frac{\text{Number of syn new mutations}}{\text{Number of syn sites}}},$$ where numbers of syn (synonymous) and nonsynonymous new mutations are given in the previous two columns of the table and number of synonymous and non-synonymous sites are calculated based on a combination of all *E. coli* protein-coding genes (see Materials and Methods). dN/dS values could not be calculated for time points in which no synonymous substitutions were observed.

[b]χ^2 P value with which it is possible to reject the null hypothesis that there is no enrichment in nonsynonymous or synonymous mutations, relative random expectations based on the number of nonsynonymous and synonymous sites within *Escherichia coli* protein-coding genes.

accumulate mutations up to 3 years under LTSP. Mutation accumulation rates appear to be fairly consistent across populations (fig. 1C). We find a significant enrichment in nonsynonymous, relative synonymous substitutions (dN/dS >1) for nonmutators across all time points up to and including day 1,095 (table 1). Such enrichment in nonsynonymous mutations is considered a hallmark of positive selection (Graur and Wen-Hsiung 2000; Ostrow et al. 2014; Tenaillon et al. 2016). Thus, our results show that bacteria are continuously adapting through mutation accumulation, during the first three years under LTSP.

Mutations accumulated up to three years under LTSP accumulate in an extremely convergent manner across the independent LTSP populations. There are 26 genes or intergenic regions, which we found to be mutated within all five populations, 20 mutated in four of the five, and 52 mutated in three of the five (supplementary table S4, Supplementary Material online). The loci mutated in 3, 4, or 5 populations constitute only 1.5%, 0.6%, and 0.7% of the *E. coli* genome, respectively. Yet, a majority of mutations occurring within nonmutators belonging to each of the five populations fall within these convergently mutated loci (fig. 2A). Since convergence constitutes a signal of positive selection (Christin et al. 2010), these results again indicate that the majority of mutations accumulated up to three years under LTSP, within nonmutator clones, are likely adaptive.

The convergently mutated genes include ones encoding some of the most central master regulators of gene expression (e.g., the RNA polymerase core enzyme, RNA polymerase's housekeeping sigma factor RpoD [σ70], the cAMP-activated global transcriptional regulator CRP, and the translation elongation factor FusA). In addition to being enriched for functions related to the regulation of gene expression, the genes that are mutated in a convergent manner across populations also include many genes related to metabolism and to transport (supplementary table S4, Supplementary Material online). We also identified a convergent event that occurred in three of the five populations and involved the deletion of 29 genes, mediated by an insertion sequence (supplementary table S4, Supplementary Material online).

Patterns of Convergence Are Suggestive of Whether Specific Genes Undergo Changes in Their Function or Loss of Function in an Adaptive Manner

The most striking example of convergence we reported in our previous study involved mutations falling within the RNA polymerase core enzyme (RNAPC). We reported that across all populations ~90% of clones carried a mutation within the RNAPC and that remarkably in the vast majority of cases this mutation fell within one of only three specific sites of the enzyme complex: RpoB position 1272, rpoC position 334, or RpoC position 428 (Avrani et al. 2017). Across the three years of the experiment, the vast majority of clones sequenced continue to carry mutations within the RNAPC that most frequently fall within one of these three sites (fig. 2B). A fourth site (RpoB position 1237) is also mutated within four of the five populations (fig. 2B). Such convergent changes to specific sites of the RNAPC indicate that specific changes to RNAPC function are adaptive under LTSP.

Interestingly, many clones develop additional mutations within the RNAPC at later time points (supplementary table S5, Supplementary Material online). These mutations only occur within clones that already carry one of the four "primary" RNAPC mutations. It is tempting to speculate that these "secondary" RNAPC mutations may be compensatory in nature. In other words, it is possible that the initial RNAPC adaptations carry a beneficial effect on fitness under LTSP, but also incur additional deleterious effects and that the secondary mutations reduce these undesirable effects. A second possibility is that as time progresses under LTSP, additional changes to RNAPC function may become advantageous leading to additional mutations within this gene complex. RNAPC mutations were demonstrated to be involved in adaptation to an intriguingly large number of different selective pressures (Conrad et al. 2010; Tenaillon et al. 2012; Avrani et al. 2017; Hershberg 2017). Further studies will be needed to ascertain exactly what these adaptations do to enhance bacterial fitness under LTSP.

We identified five additional examples in which only one or a few sites of a specific locus were mutated in a highly
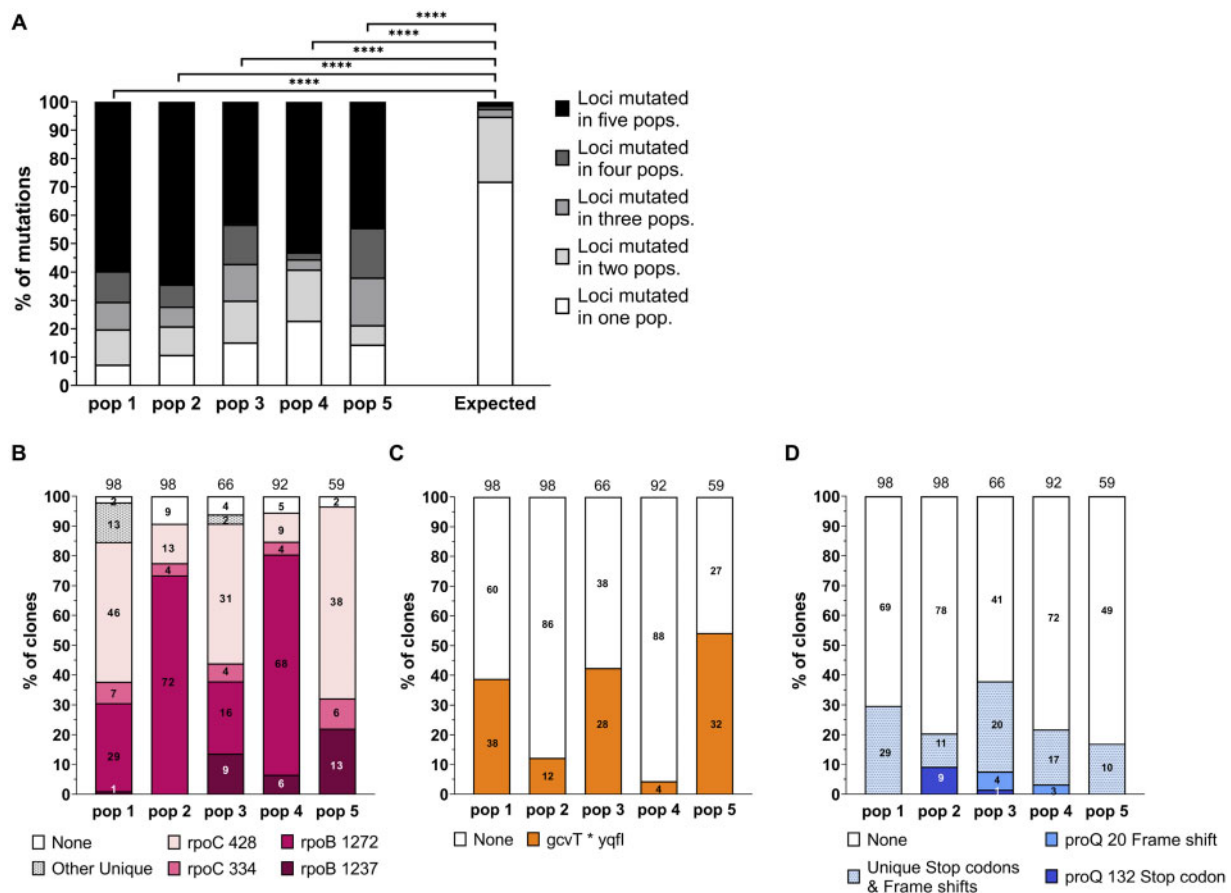
Fig. 2. Highly convergent patterns of mutation accumulation under LTSP. (A) The majority of mutations occurring within nonmutator LTSP clones occur within genes that are mutated in a convergent manner. For each population, the number of mutations falling within genes mutated in 1, 2, 3, 4, or all 5 populations is presented. The relative length of all genes mutated in 1, 2, 3, 4, or all 5 of the populations was used to draw the expected bar, which represents random expectations. **** denotes a statistically significant difference ($P < 0.0001$, according to a χ^2 test). (B) Convergent mutations within the RNA polymerase core enzyme. Depicted is the proportion of clones carrying mutations within each mutated RpoB and RpoC position (for each population). (C) gcvT promoter/5′ UTR region as an example of loci containing one or at most two specific convergent mutations. All five examples of such loci are presented in supplementary figure S1, Supplementary Material online. (D) proQ, as an example of genes that are deactivated in a convergent manner across populations. All five examples of such genes are presented in supplementary figure S2, Supplementary Material online. For the (B)–(D), positions containing a mutation in more than one population are given their own designation. Such positions are marked by the protein name and the position number. If the mutation occurring is a frame shift or stop codon, this is indicated. Positions that are mutated in only a single population are grouped within the "other unique" section of their population's bar, if they result in a nonsynonymous substitution and in the "unique stop codons and frame shift" section, if they result in a stop codon or frame shift.

convergent manner and at very high frequencies across populations (a single example is presented in fig. 2C, all five examples are presented in supplementary fig. S1, Supplementary Material online). Of the five examples, three occurred within the promoter/5′ UTR regions of genes: A specific mutation within the promoter region of the glycine cleavage system gene gcvT, which disrupts the promoter's known σ70-35 hexamer (Santos-Zavaleta et al. 2019), appears at substantial frequencies across all five populations (fig. 2C). This is the only mutation seen within the locus, highlighting the likelihood that highly specific changes to the expression of gcvT are adaptive under LTSP. The promoter region of the alcohol dehydrogenase gene adhP also contains a specific mutation across all five populations (supplementary fig. S1B, Supplementary Material online). Finally, the promoter/5′ UTR of the transporter gene cycA is mutated at high frequencies across all five populations. In the

majority of cases one of two mutations occur within this locus (supplementary fig. S1D, Supplementary Material online). One of these two mutations appears in four of the populations and is located 43 bases upstream to the gene's transcription start site (Santos-Zavaleta et al. 2019). The second of these mutations appears in three of the five populations and falls within a known binding site of the small RNA GcvB, which regulates the expression of cycA at the posttranscriptional level (Santos-Zavaleta et al. 2019). Specific high frequency convergent mutations are also observed within the genes dppA (supplementary fig. S1C, Supplementary Material online) and sstT (supplementary fig. S1E, Supplementary Material online). As with RNAPC, the fact that we observe very specific sites to be mutated in a convergent manner across populations indicates that it is specific changes to the expression or function of these five genes that are adaptive under LTSP.
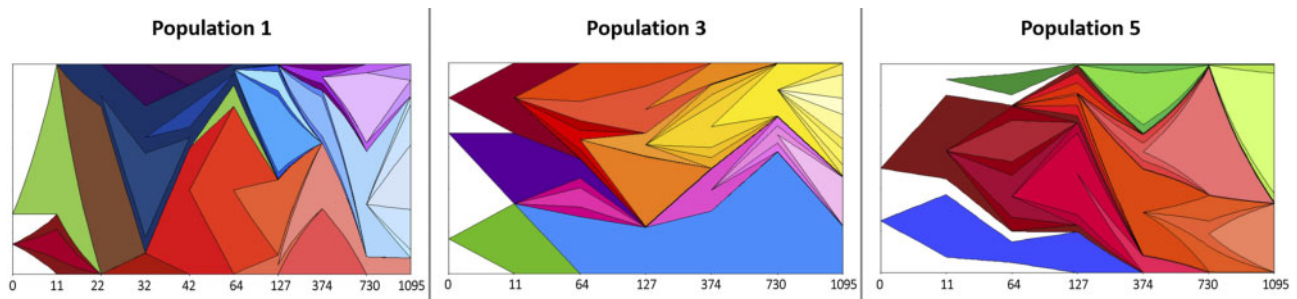
FIG. 3. Populations maintain high levels of genetic variation up to three years under LTSP. A clear pattern of clonal interference by which several genotype compete for dominance across time points can be seen. Muller diagrams depicting the relative frequencies of different haplotypes segregating within LTSP populations 1, 3, and 5 are presented. The x-axis indicates the sampling times (not to scale). Only mutations appearing in 30% or more of the considered population's clones, during at least one time point, were used to generate each plot. In population 3, which included mutator clones once the mutator lineage emerged, we do not depict the variation occurring within it, as it is too extensive to draw accurately. This lineage is represented in blue in the population 3. Due to very high frequencies of mutator clones within populations 2 and 4, we do not present Muller plots for these populations. Muller plots were produced using the R package MullerPlot (Farahpour et al. 2016).

In total, we observed 23 specific convergent mutations, each present across three or more of the five independently evolving LTSP populations (supplementary table S6, Supplementary Material online).

For five additional genes, we found that across populations deactivating mutations (inserting stops codons or frameshifts) tended to occur (A single example is presented in fig. 2D, all five examples are presented in supplementary fig. S2, Supplementary Material online). These genes were the RNA chaperone gene *proQ* (fig. 2D), the transporter genes *glpF* and *dcuA*, the ribosomal protein acetyltransferase gene *rimJ*, and the transcriptional repressor gene *paaX* (supplementary fig. S2, Supplementary Material online). The convergent occurrence of deactivating mutations within these genes suggests that it may be adaptive to remove the function of these specific genes under LTSP.

Populations Adapting under LTSP Maintain High Levels of Standing Genetic Variation throughout the First three Years of Adaptation

Despite the high level of convergence with which adaptations occur between independently evolving LTSP populations, we find that adaptive alleles never fix across any entire individual population. Instead multiple genotypes tend to compete for dominance across all populations and time points, in a pattern of soft sweeps (fig. 3). As a result, LTSP populations continuously maintain within them very high levels of genetic variation, even as they adapt in a highly convergent manner to prolonged resource exhaustion. The pattern of soft sweeps, which is observed when examining data from nonmutators, suggests that, even for them, adaptation continues to not be limited by mutational input, up to three years under resource exhaustion. Further studies will be required in order to establish whether the maintenance of multiple genotypes over very long periods of time under LTSP is driven by balancing selection, clonal interference, or by other processes.

Combined, we observe both mutation accumulation with time and fluctuations in the frequencies of genotypes, as cell numbers remain stable over long periods of time. This strongly suggests that cellular replication continues under LTSP, up to three years into these experiments.

Continuous Maintenance of Variation in Mutation Rates within Mutator-Containing LTSP Populations

As already mentioned, we observed within three of the five LTSP populations the emergence of mutator clones, which acquired a mutation within a mismatch repair gene (supplementary table S2, Supplementary Material online). In populations 2 and 3, mutator clones carried mutations within the *mutS* gene. In population 4, a majority of mutators carried a mutation within *mutL*, whereas a minority carried a mutation within the gene *mutH*. Interestingly, the three populations in which mutator clones were observed by day 64 of our experiments, continued to include such clones, at observable frequencies, up to three years into the experiment (fig. 4A–C). In contrast, in the two populations in which such clones were not observed by day 64, no such clones were observed up to three years into our experiments. Five populations are not, in our opinion, sufficient to be certain that this pattern by which mutators emerge under LTSP early or not at all is a general trend. However, in the future, it might be interesting to examine whether this is indeed a general trend through the establishment of additional LTSP populations.

In population 3, mutators and nonmutator clones coexist at high frequencies up to 3 years under LTSP (fig. 4B). In populations 2 and 4, mutator clones rose to very high frequencies, leading to us sequencing only such clones at day 1,095 of the experiment. However, we did observe, out of the approximately ten clones sequenced, one nonmutator clone in each of these populations at day 730 (fig. 4A and C). We can therefore conclude that mutator and nonmutator clones tend to coexist for long periods of time under LTSP. It is important to note that while we did not observe any nonmutator clones in populations 2 and 4 at day 1,095, this does not mean that they are necessarily not present within the population. After all, by sequencing approximately ten clones
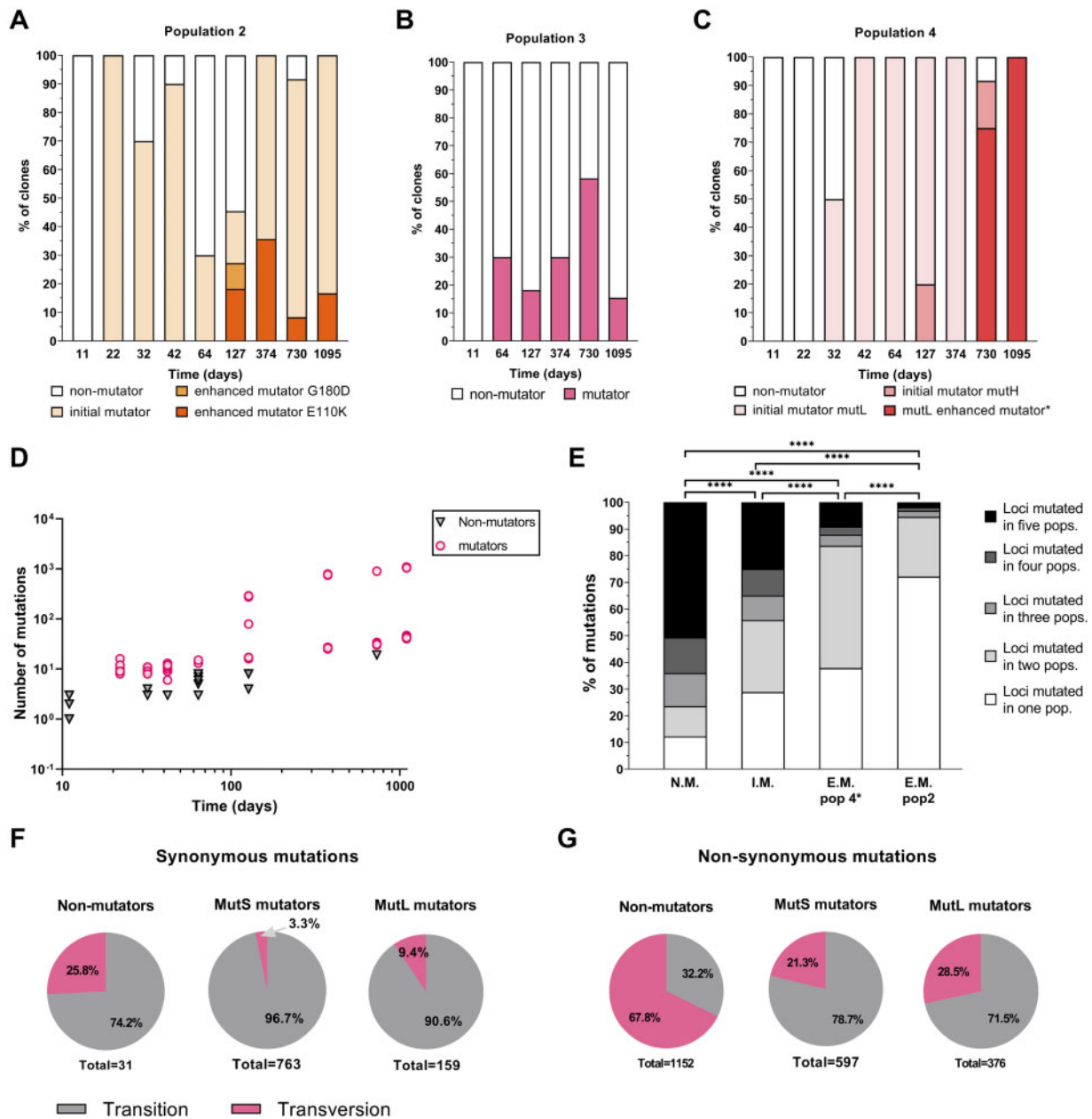
Fig. 4. Dynamics of mutator evolution under LTSP. (*A–C*) Clones that vary in their mutation rates coexist for long periods of time under resource exhaustion in all LTSP populations in which mutators evolved. For each population, the relative frequency of each type of clone present within that population is depicted. In population 4, *mutL* clones sampled from days 730 and 1,095 are marked using a different color from *mutL* clones extracted at earlier time points, due to their larger than expected mutation accumulation. It is, however, important to note that we did not observe a significantly higher frequency of development of rif resistance within *mutL* clones extracted during or after day 730 and *mutL* clones extracted prior to day 730. (*D*) Enhanced mutation rate mutators emerge within population 2. Numbers of mutations accumulated by individual population 2 clones, as a function of time spent under LTSP. Each mark represents an individual clone. Nonmutator clones are represented by black triangles. Mutator clones are represented by magenta dots. Both axes are presented on a logarithmic scale. (*E*) Higher fractions of mutations fall within convergently mutated genes in clones with lower mutation rates. For each type of clone, the number of mutations falling within genes mutated in 1, 2, 3, 4, or all 5 populations is presented. **** denotes a statistically significant difference between bars ($P < 0.0001$, according to a χ^2 test). (*F*) Higher transition bias of synonymous mutations, counted only once, within mutators compared with nonmutators. (*G*) Higher transition bias of nonsynonymous substitutions occurring within convergently mutated genes, within mutators compared with nonmutators. For (*F*) and (*G*), numbers below each pie chart indicate the total numbers of mutations used to calculate the percentages presented in that pie chart.

out of millions of cells present within each population, we can only hope to identify the most frequent genotypes.

In population 2, mutator clones, carrying a MutS T300K mutation were first observed at day 22. Up till day 64 clones carrying the T300K MutS mutations had a rather similar number of mutations to each other, which ranged on the order of twice as many mutations as clones that did not carry a mutator mutation (supplementary table S3, Supplementary Material online). However, at day 127, mutators seemed to diverge into three clusters (fig. 4D). Although two of the sequenced

mutator clones followed the trajectory of mutation accumulation observed for these mutators so far (17 mutations/clone), three of them accumulated a much higher number of mutations than would be expected from this trend (accumulating 79, 275, and 290 mutations, fig. 4D). All mutator clones, irrespective of their accumulated number of mutations, carried the same T300K MutS mismatch repair mutation. We will refer from now on to those mutators carrying a higher number of mutations as "enhanced" mutators. In contrast, mutators carrying a lower number of mutations, consistent with the initial rate of mutations accumulation within mutators, will be referred to as "initial" mutators. Within population 2, enhanced mutators were also observed at the later time points of our experiments, but always coexisted with initial mutators (fig. 4A and D).

In order to attempt and identify more of the clones with the enhanced mutator phenotype and examine whether these contain a secondary mutation explaining their higher mutation rate, we sequenced five additional clones from population 2 day 127 after plating clones on plates containing the antibiotics rifampicin (rif) or nalidixic acid. By sequencing clones that developed antibiotic resistance we hoped to enrich for clones that acquired a larger number of mutations overall. Indeed, all five antibiotic-resistant clones sequenced carried the MutS T300K mutator mutation, and each acquired a total of 52, 59, 303, 342, and 609 mutations, placing them in the enhanced mutator category. All 16 enhanced mutator clones identified throughout the experiment contained a mutation within the gene *dnaQ*. In contrast, none of the 60 initial mutator clones carry such a mutation. The *dnaQ* gene encodes the epsilon subunit of DNA polymerase III, which is a 3′–5′ exonuclease responsible for proofreading DNA replication. It was previously shown that mutations within *dnaQ* can greatly increase mutation rates (Echols et al. 1983; Fijalkowska and Schaaper 1996). Intriguingly, the three day 127 clones that accumulated between 52 and 79 mutations carried a G180D DnaQ mutation, whereas the five that accumulated over 275 mutations by day 127 suffered a different mutation within DnaQ (E110K). At later time points only the E110K mutation was observed, fitting with the higher mutation accumulation rates of all enhanced mutator clones sequenced from day 374 onward (fig. 4D).

Next, we wanted to independently verify that the different types of mutators identified in population 2 indeed differ in their mutation rates, as expected from their patterns of mutation accumulation. To do so, the frequencies with which the four types of clones segregating within population 2 develop resistance to the antibiotic rif, following overnight growth in fresh LB without rif, was compared (supplementary fig. S3, Supplementary Material online). As expected, nonmutator clones developed resistance at the lowest average frequency (3.5×10^{-9}), compared with initial mutators, which developed resistance at a higher average frequency of 5.8×10^{-7}. The G180D DnaQ enhanced mutators developed resistance at an average frequency higher than that of the initial mutators (4.8×10^{-6}), but lower than that of the E110K DnaQ enhanced mutators (3.7×10^{-5}). All reported

differences were statistically significant according to a non-paired, one tailed Mann–Whitney test ($P < 0.01$ for all comparisons). Thus, it appears that fitting with rates of mutation accumulation under LTSP, the three mutator types evolving within population 2 indeed vary in their mutation rates, with initial mutators, which were the first to emerge having the lowest mutation rates and the E110K DnaQ enhanced mutators having the highest.

Although mutators and nonmutators coexist within population 3 throughout the examined time points, at the final time point, the two sequenced mutators seemed to have acquired fewer mutations than we would expect from previous trends (supplementary fig. S4, Supplementary Material online). These results were somewhat suspect given the fact that the two sequenced mutator clones had relatively low-sequencing coverage (supplementary table S1, Supplementary Material online). To further examine this, we examined the rates with which nonmutator and mutator population 3 clones extracted from the final time point (day 1,095), and from the previous time point (day 730) develop resistance to the antibiotic rif, following overnight growth in fresh LB without rif (supplementary fig. S3, Supplementary Material online). We found that mutator clones extracted at both time points had a similar rate of rif resistance that was significantly higher than the rate observed for the nonmutators. Our results thus suggest that mutators and nonmutators coexist within population 3, up to at least three years under LTSP.

Different types of mutators coexist over time within population 4. However, in this population, the differences in mutation rates between the mutator types seem to be less extensive. In population 4, we first observed mutators carrying a mutation within the mismatch repair gene *mutL* at day 32. At day 127, we observed the *mutL* mutators alongside a second type of mutators carrying a mutation within *mutH*. At day 127, both *mutL* mutators and *mutH* mutators carried a rather similar number of mutations (supplementary fig. S5, Supplementary Material online), which was consistent with the number of mutations observed for the initial mutators within populations 2 and 3 at day 127 (supplementary fig. S6, Supplementary Material online). At day 374 we observed only the *mutL* mutators and these again carried a number of mutations which was consistent with that observed within initial mutators within populations 2 and 3 (supplementary figs. S5 and S6, Supplementary Material online). However, at day 730, we again observed both *mutL* and *mutH* mutators. While the *mutH* mutators seemed to accumulate mutations at a rate consistent with initial mutators, the *mutL* mutators acquired more than three times as many mutations, indicating that they may have increased their mutation rate further (supplementary fig. S3, Supplementary Material online). At day 1,095, only the putative enhanced mutator *mutL* clones were observed.

To further compare the mutation rates of the different types of mutators found within population 4, we characterized the frequency with which four types of population 4 clones develop resistance to the antibiotic rif following overnight growth in the absence of that antibiotic (supplementary

Table 2. Enrichment of Nonsynonymous or Synonymous Mutations, Relative Random Expectations, within Different Clone Types.

Type of Clones	Number of Nonsynonymous Substitutions	Number of Synonymous Substitutions	dN/dS[a]	P value[b]
Nonmutators	1,224	116	3.24	<0.0001
Initial mutators	1,227	263	1.43	<0.0001
Population 4 enhanced mutators	1,269	331	1.18	0.0076
Population 2 enhanced mutators	2,894	1,583	0.56	<0.0001

[a]dN/dS (the ratio of the rates of nonsynonymous to synonymous mutations) was calculated as follows:

$\frac{dN}{dS} = \frac{\frac{\text{Number of non-syn new mutations}}{\text{Number of non-syn sites}}}{\frac{\text{Number of syn new mutations}}{\text{Number of syn sites}}}$, where numbers of syn (synonymous) and nonsynonymous new mutations are given in the previous two columns of the table and number of synonymous and nonsynonymous sites are calculated based on a combination of all *Escherichia coli* protein-coding genes (see Materials and Methods).
[b]χ^2 P value with which it is possible to reject the null hypothesis that there is no enrichment in nonsynonymous or synonymous mutations, relative random expectations based on the number of nonsynonymous and synonymous sites within *Escherichia coli* protein-coding genes.

fig. S3, Supplementary Material online). We found that a non-mutator clone extracted from population 4 at day 730 developed resistance to rif at an average frequency of 5.9×10^{-10}. The *mutH* clone extracted from the same population and time point developed resistance to rif at significantly higher frequencies (2.4×10^{-7} on average, $P < 0.001$, according to a nonpaired one-tailed Mann–Whitney test). Although the *mutH* clone used for this analysis accumulated 31 mutations by day 730, the *mutL* clone we used, which was extracted from the same time point, accumulated 114 mutations. Fitting with this, the *mutL* clone developed rif resistance following overnight growth at significantly higher frequencies, compared with the *mutH* mutator clone (3.8×10^{-6}, $P < 0.001$). Based on the relative jump in the numbers of mutations accumulated by the *mutL* mutator clones, between days 374 and 730, we expected that the *mutL* clones may have developed a higher mutation rate during that time. However, no significant difference was found between the frequencies with which the day 374 and a day 730 *mutL* clones we examined developed resistance to rif ($P = 0.215$).

Despite Their Acquiring a Substantial Deleterious Burden, Clones with Very High Mutation Rates Can Persist for Prolonged Periods of Time, Alongside Clones with Lower Mutation Rates

We find that ratios of the rates of nonsynonymous to synonymous substitutions accumulated by nonmutators, initial mutators, and population 4 putative-enhanced mutators are all significantly higher than one (table 2). This indicates that within these three clone types mutation accumulation tends to be dominated by positive selection. Further supporting this is the high fraction of mutations accumulated within such clones that fall within genes that are mutated across multiple populations (fig. 4E). Positive selection appears to be affecting mutation accumulation more strongly within nonmutators than within initial mutators. This is reflected by significantly higher dN/dS values ($P < 0.0001$, according to a χ^2 test) and by the fact that within nonmutators significantly higher fractions of mutations tend to fall within genes mutated across larger numbers of populations (fig. 4E, $P < 0.0001$, according to a χ^2 test). In sharp contrast, the vast majority of mutations found within population 2 enhanced mutator clones occur within genes that are not

mutated in any of the other populations (fig. 4E). Furthermore, mutations accumulated by the population 2 enhanced mutators are significantly enriched for synonymous substitutions, relative random expectations (dN/dS < 1, table 2). This constitutes a signal of purifying selection dominating patterns of mutation accumulation (Graur and Wen-Hsiung 2000). In other words, population 2 enhanced mutators acquire a substantial number of deleterious mutations due to their extremely high mutation rates. Indeed, in order to observe such a dN/dS, which is much lower than 1, many cells of this type would have had to be purged from the population by selection. The extremely high mutation rate of the population 2 enhanced mutators therefore seems to impose on them a high deleterious burden. Yet, despite this deleterious burden, they persist alongside nonmutators (at least up till day 730) and alongside initial mutators (at least up to day 1,095), within population 2 (fig. 4A).

What advantage could population 2 enhanced mutator clones carry that enables them to persist alongside lower mutation rate clones, despite their deleterious burden? One possibility is that the higher mutation rate of these clones may enable them to accumulate higher numbers of adaptive mutations, counterbalancing the deleterious effects of mutation accumulation. As described above, we identified 98 loci that are mutated in a convergent manner across three or more of the five LTSP populations. The mutations observed within our clones that fall within these loci are likely to be adaptive under LTSP. By day 1,095 of our experiments, nonmutator and initial mutators accumulated mutations within 17.1% and 16.2% of these genes, on average, respectively. At the same time, the population 2 enhanced mutator clones accumulated mutations within 48.5% of the convergently mutated genes, on average. Thus it appears that while the higher mutation rates of the population 2 enhanced mutators led to a higher accumulation of deleterious mutations, it also enabled these clones to acquire a higher number of adaptive mutations.

A previous study by Gentile et al. showed that genotypes carrying two mutator alleles (similar to our population 2 enhanced mutators) can outcompete genotypes bearing a single mutator allele (similar to our initial mutators) (Gentile et al. 2011). However, to our knowledge, we are the first to demonstrate such an event arising spontaneously within the context of an evolutionary experiment. Indeed, the opposite

trend is often expected by which following the emergence of a mutator, antimutator alleles will tend to shift rates of mutation back down (Couce and Tenaillon 2019). For example, in Richard Lenski's long-term evolutionary experiment (LTEE), one of the populations was found to evolve a *mutT* mutator phenotype, increasing mutation rates by ∼150 fold. This population was later invaded by a *mutY* antimutator allele, which decreased mutation rates by ∼40–60% (Wielgoss et al. 2013). It was suggested that this occurred because the supply of adaptive mutations reduced once the LTEE populations became better adapted to the conditions imposed on them, which remain constant with time under the LTEE's experimental design. Indeed, in the LTEE the greatest fitness gains occurred early on, with a later pattern of diminishing returns (Lenski and Travisano 1994). The fact that in our experiment, we see mutators with higher mutation rates coexisting at high frequencies alongside those with lower mutation rates may be the result of adaptive mutation availability remaining high up to three years under LTSP. One possibility for why adaptive mutations may continue to be available under LTSP is that conditions might be changing, requiring cells to continuously adapt to these changes. A second possibility is that conditions remain fairly constant, but that the ancestral *E. coli* genotype is initially far from the fitness peak for these conditions. More research will be needed in order to distinguish between these two possibilities.

Mutator Evolution Can Affect Both Mutational Spectra and the Spectrum of Adaptive Substitutions

In addition to affecting mutation rates, mutator mutations also have the potential to affect mutational spectra (i.e., which types of mutations will tend to occur more or less frequently). In order to estimate mutational spectra, it is necessary to examine mutations accumulated in the absence of selection (Hershberg and Petrov 2010). However, mutations accumulated under LTSP are strongly affected by selection. We therefore attempted to examine mutational spectra by focusing on synonymous mutations, which are less likely to be subject to strong selection and by considering each synonymous mutation only once, irrespective of the number of clones it appeared in. For nonmutator clones, ∼74% of these synonymous mutations were transitions (i.e., mutations from a purine to a purine or from a pyrimidine to a pyrimidine, fig. 4F). The transition bias was even more pronounced within mutators, where over 90% of mutations were transitions (fig. 4F). This observed enrichment in transition mutations within mutators was previously demonstrated for a *mutL* mutator in a mutation accumulation study (Lee et al. 2012). Such an increased transition bias may be expected to occur within mutators that are defective in their mismatch repair genes. After all, mismatch repair genes are responsible for repairing errors due to deamination, which tends to lead to C/G to T/A transitions (Zell and Fritz 1987).

Due to the structure of the genetic code, transitions are less likely than transversions to lead to nonsynonymous substitutions. The increased transition bias of mutators therefore likely decreases their accumulation of protein-disruptive mutations and by doing so may decrease the deleterious burden they experience. This might contribute to the ability of mutators to persist within our populations alongside nonmutator clones. This fits with a recent study that found that differences in mutational biases between different mutators can lead to variation in the proportion of mutations they suffer that lead to disruption in protein sequences. Computer simulations showed that such differences may potentially greatly affect the strength of selection to decrease the mutators' mutation rates (Couce and Tenaillon 2019). However, it is important to note that at least for the population 2 enhanced mutators, we do find evidence that their extremely high mutation rates do lead to a substantial deleterious burden, and that despite this burden they persist for long periods of time, alongside clones with much lower mutation rates.

Since convergence is a strong signal of positive selection, nonsynonymous mutations falling within convergently mutated genes are very likely to be adaptive. It is therefore possible to look at such mutations to study the spectra of adaptive substitutions. As expected from the structure of the genetic code, we find that across clone types, nonsynonymous substitutions occurring within convergently mutated genes are more frequently the result of transversion mutations, compared with the synonymous substitutions we examined above. At the same time, nonsynonymous mutations within convergently mutated genes are enriched for transitions within mutators compared with nonmutators (fig. 4G). This suggests that differences in mutational biases between mutators and nonmutators translate into differences in the types of adaptive substitutions that ultimately occur.

Conclusions

We show that *E. coli* populations genetically adapt in a continuous manner up to at least three years under LTSP. Patterns of adaptation are remarkably convergent, with large majorities of nonmutator mutations falling within loci that are mutated across most or all independently evolving populations. This in combination with strong enrichment in nonsynonymous versus synonymous mutations within protein-coding genes suggests very strong positive selection affecting mutation accumulation under LTSP. Despite this strong selection LTSP populations continue to maintain very high levels of genetic variation under LTSP, with no single lineage fixing at any given time point, up to and including three years into the experiments. This suggests that despite their existing within spent media for years, adaptation of LTSP populations is not limited by mutational input, even within nonmutators.

Within three of the five examined populations, we saw the emergence of mutator clones fairly early on. Once they emerged, mutators were able to coexist alongside nonmutators for very long periods of time. In two of the three mutator-containing populations, we observed the evolution of mutators with substantially different mutation rates that then coexisted over long time frames. This trend was most

pronounced in population 2, where the acquisition of secondary mutation rate-enhancing mutation on the background of an existing mismatch repair mutation, led to the emergence of enhanced mutators with extremely high mutation rates. These enhanced mutators then coexisted alongside nonmutators and initial mutators over long periods of time, despite the fact that they suffered a high deleterious burden. The deleterious burden suffered by the enhanced mutators may have been counter-balanced by their ability to accumulate a much higher number of LTSP adaptations. Our results demonstrate that under strong selection, it is possible to observe the emergence and long-term coexistence of clones with very different mutation rates and associated deleterious burdens.

Finally, we show that in addition to affecting overall mutation accumulation rates, mutator mutations can also affect mutational biases and types of ultimate adaptive substitutions that emerge, as result of these biases. Although overall mutation accumulation rates within mutators are substantially higher, the mutations accumulated tend to be enriched for transitions, which have a lower likelihood of disrupting protein sequences. This may enable mutators to decrease their associated deleterious burden.

Materials and Methods

LTSP Evolutionary Experiments

Five LTSP populations were initiated in July 2015, as described in our previous paper describing the first four months of these experiments (Avrani et al. 2017). Briefly, each population was initiated from a separate clone of *E. coli* K12 strain MG1655. $\sim 5 \times 10^6$ cells/ml were inoculated into 400 ml of LB within a 2 l polycarbonate breathing flask. The five flasks were placed in an incubator set at 37 °C, where they have been shaking ever since at 225 rpm. No new nutrients or resources were added to the cultures with time, except for sterile water that is added to compensate for evaporation every 10–15 days, according to the weight lost by each flask during that time period. Periodically, every few months, as small cracks appeared in the polycarbonate flasks, we transferred the populations into new flasks. At the time of transfer, no visible biofilm or wall growth was observed.

Sampling LTSP Populations and Estimating Viability

Initially, every day, then every week, then every month, and following that at longer intervals (fig. 1), 1 ml of each culture was sampled. Dilutions were plated using a robotic plater to evaluate viability through live counts. Samples were frozen in 50% glycerol at −80 °C.

Sequencing of LTSP Clones

Frozen cultures from each of the populations and time points (days 374, 730, and 1,095) were thawed and dilutions were plated and grown over night. Approximately ten colonies (supplementary table S1, Supplementary Material online) from each culture were used to inoculate 4 ml of medium in a test tube and were grown until they reached an optical

density of ~ 1. This procedure was followed in order to minimize the number of generations each clone undergoes prior to sequencing and thus minimize the occurrence of mutations during regrowth. One milliliter of the culture was centrifuged at $10,000 \times g$ for 5 min and the pellet was used for DNA extraction. The remainder of each culture was then archived by freezing in 50% of glycerol at −80 °C. DNA was extracted using the Macherey-Nagel NucleoSpin 96 Tissue Kit. Library preparation followed the protocol outlined in Baym et al. (2015). Sequencing was carried out at the Technion Genome Center using an Illumina HiSeq 2500 machine. Clones were sequenced using paired end 150 bp reads.

The ancestral clones used to initiate the five populations were sequenced as part of our previous study as were approximately ten clones from each of the five LTSP populations at days 11, 64, and 127 and approximately ten clones from days 22, 32, and 42 from populations 1, 2, and 4 (supplementary table S1, Supplementary Material online) (Avrani et al. 2017).

Calling of Mutations

In order to call mutations, the reads obtained for each LTSP clone were aligned to the *E. coli* K12 MG1655 reference genome (accession NC_000913). LTSP clone mutations were then recorded if they appear within an LTSP clone's genome, but not within the ancestral genome, which was previously sequenced (Avrani et al. 2017). Alignment and mutation calling were carried out using the Breseq platform, which allows for the identification of point mutations, short insertions and deletions, larger deletions, and the creation of new junctions (Deatherage and Barrick 2014).

Quantifying Rif Resistance Frequencies for Individual LTSP Clones Following Overnight Growth in Fresh LB

Clones were taken from −80 °C storage and streaked to isolate an individual random colony. The colony was inoculated into 4 ml of LB for overnight incubation at 37 °C with shaking (225 rpm). Following overnight growth, 100 μl of the resulting culture was plated on LB agar plates and on LB agar plates supplemented with 100 μg/ml rif (Sigma–Aldrich) at the appropriate dilutions. Frequencies of rif resistance were calculated by dividing the CFUs grown on rif-containing plates by the CFUs grown on plates with no rif added. For each clone tested at least five independent experiments were carried out.

Calculating Numbers of Synonymous and Nonsynonymous Sites within the *E. coli* K12 MG1655 Genome

DNA sequences of all protein-coding genes of *E. coli* K12 MG1655 were downloaded from the NCBI database. The contribution of each protein-coding site to the count of nonsynonymous and synonymous sites was calculated according to the likelihood that mutations to that site would lead to a nonsynonymous or a synonymous change. For example, mutations to the third codon position of a 4-fold degenerate codon would be 100% likely to be synonymous. Such a

position would therefore add a count of one to the number of synonymous sites. In contrast mutations to the third codon position of a 2-fold-degenerate codon will be synonymous for a third of possible mutations and nonsynonymous for two-third of possible mutations. Such positions would therefore add a count of one-third to the number of synonymous sites and two-third to the number of nonsynonymous sites. In such a manner, we could calculate what proportion of sites, across all *E. coli* K12 MG1655 protein-coding genes are non-synonymous (meaning that mutations to those sites would lead to nonsynonymous changes) and what proportion are synonymous.

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

Acknowledgments

Data Availability

The raw sequencing reads for the clones sequenced as part of the current study have been deposited in the Short Read Archive, under BioProject ID: PRJNA674613. Raw sequence data for clones previously sequenced from the first four months of the experiments are deposited under BioProject ID: PRJNA380864.

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