Culture Volume Influences the Dynamics of Adaptation under Long-Term Stationary Phase

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Abstract

*Escherichia coli and many other bacterial species, which are incapable of sporulation, can nevertheless survive within resource exhausted media by entering a state termed long-term stationary phase (LTSP). We have previously shown that *E. coli* populations adapt genetically under LTSP in an extremely convergent manner. Here, we examine how the dynamics of LTSP genetic adaptation are influenced by varying a single parameter of the experiment—culture volume. We find that culture volume affects survival under LTSP, with viable counts decreasing as volumes increase. Across all volumes, mutations accumulate with time, and the majority of mutations accumulated demonstrate signals of being adaptive. However, positive selection appears to affect mutation accumulation more strongly at higher, compared with lower volumes. Finally, we find that several similar genes are likely involved in adaptation across volumes. However, the specific mutations within these genes that contribute to adaptation can vary in a consistent manner. Combined, our results demonstrate how varying a single parameter of an evolutionary experiment can substantially influence the dynamics of observed adaptation.

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

Significance

Evolutionary experiments are highly useful in that they enable us to study the dynamics of adaptation as it occurs. However, it is often hard to know which aspects of these dynamics are more general, and which more specific to the experimental design used. Here, we address this question by adjusting a single parameter of an evolutionary experiment designed to study the dynamics of adaptation under LTSP. Our results advance both our understanding of the general dynamics of evolution under LTSP and of the manner in which altering a single parameter of an evolutionary experiment can affect the patterns of adaptation observed.

Introduction

*Escherichia coli* is capable of surviving for months and even years within batch culture, without the addition of any external growth resources, in a state termed long-term stationary phase (LTSP) (Zambrano and Kolter 1993; Zambrano et al. 1993; Finkel and Kolter 1999; Liao and Shollenberger 2003; Finkel 2006; Rozen et al. 2009; Avrani et al. 2017). When placed into fresh rich media, *E. coli* will undergo a short period of exponential growth, followed by a short stationary phase and a rapid death phase. Death phase does not, however, lead to the extinction of the entire *E. coli* population. Instead, populations can enter LTSP and maintain viability over many months and even years. We have previously published a paper describing the dynamics of adaptation during the first 4 months spent under LTSP (Avrani et al. 2017). We found that *E. coli* LTSP populations adapt genetically through the rapid acquisition of mutations. These mutations are enriched for functional categories,
relative to random expectations indicating that positive selection strongly affects their accumulation. Further evidence for the adaptive nature of mutation accumulation is the extreme convergence with which mutation accumulation occurs. The most striking example of convergent mutation accumulation under LTSP involves the RNA polymerase core enzyme (RNAPC). We found that across all populations and time points sampled ~90% of the hundreds of sequenced clones carry a mutation within one of two of the genes encoding the RNAPC, rpoB and rpoC. For >90% of the clones carrying an RNAPC mutation, we found that one of only three specific sites was mutated: RpoB position 1,272, RpoC position 334, or RpoC position 428. Clones containing mutations within each of these positions appeared within all five independently evolving LTSP populations. Such high levels of convergence demonstrate that RNAPC mutations and particularly mutations to these three specific sites of the RNAPC are adaptive within our LTSP populations.

Previous studies into LTSP that did not sequence whole genomes, suggested that E. coli adaptation under LTSP often relies on mutations within the master regulator of the stress response, RpoS (Zambrano et al. 1993; Farrell and Finkel 2003; Finkel 2006; Battesti et al. 2011). It was therefore surprising that we saw no mutations within RpoS in our LTSP populations. One possibility for this apparent discrepancy was that the conditions under which LTSP was studied in our experiments varied from those of previous studies. Although most past studies examined adaptation to LTSP as it occurs in much smaller volumes (<5 ml) in test tubes, we examined adaptation to LTSP in large volumes (400 ml) within flasks. Indeed, it has been demonstrated that culture volume and receptacle, as well as media type can affect survival dynamics under LTSP (Riedel et al. 2013; Kram and Finkel 2014, 2015; Kram et al. 2017). Culture volume in particular can affect both the overall population size and the conditions faced by the bacteria, prior to and during LTSP.

In this study, we examine how varying culture volume affects the dynamics of genetic adaptation under LTSP. Our results suggest that manipulation of only one parameter of our experiments (growth volume), can substantially affect the dynamics of adaptation as these pertain to rates of mutation accumulation, the influence of natural selection on mutation accumulation, and the identity of loci and specific sites involved in adaptation.

Materials and Methods

LTSP Evolutionary Experiments Growth Conditions

All our experiments were carried out in flasks of the same shape, with the same relative ratio between growth media and flask sizes. To initiate a new LTSP evolutionary experiment, a single colony of E. coli K12 MG1655 was grown overnight. Single colonies were used to inoculate test tubes with 4 ml of fresh Luria Broth (LB) medium. Each culture was then grown until it reached an OD (600 nm) of 0.4 and then used to inoculate 4, 40, and 400 ml of LB in a ratio of 1:400 in 20 ml glass flasks and 200 and 2,000 ml polycarbonate breathing flasks, respectively. This resulted in the inoculation of ~10^6 cells per ml of culture (fig. 1A). This procedure was used to both start with a similar cell concentration in all populations and start with isogenic populations by reducing the generations passed since each population was a single cell. The 15 resulting flasks were placed in an incubator set at 37 °C, shaking at 225 rpm. No new nutrients or resources were added to the cultures with time, except for sterile water that was added to compensate for evaporation every day, according to the weight lost in each flask during that time. Note that no water was added to counteract sampling of the cultures. Following the final sampling point at day 64, the volume of each culture was 388, 38.8, and 3.88 ml for the initial 400, 40, and 4 ml flasks, respectively. The change in volume across each experiment was therefore lower than 5%.

Sampling LTSP Populations and Estimating Viability

One milliliter of each 400 ml culture, 100 µl of each 40 ml culture, and 10 µl of each 4 ml culture were sampled, at days 0, 1, 2, 4, 5, 7, 8, 11, 15, 22, 52, and 64 (fig. 1A). Dilutions were plated using a robotic plater to evaluate viability using live counts. Samples were frozen in 50% glycerol in a −80 °C freezer for future analyses. Samples with volumes <1 ml were diluted in LB to enable their preservation. For each sample, pH was measured using pH indicator strips.

Sequencing of LTSP Clones

Although, in this study, we initiated LTSP evolutionary experiments at three different volumes (4, 40, and 400 ml), we sequenced only two of them (4 and 40 ml) and analyzed them in comparison with the 400 ml LTSP experiments previously established in our lab (Avrani et al. 2017). (It is important to note that these previous experiments were carried out in precisely the same manner as our current 400 ml experiments were and that their growth dynamics were nearly identical to the current 400 ml experiments). Frozen cultures of each population and time point were thawed and dilutions were plated and grown overnight. Approximately 10 clones from four of the 4 and 40 ml populations (eight populations in total) were sequenced at each of three time points of the experiments: 11, 22, and 64 days (supplementary table S1, Supplementary Material online). Approximately 10 clones from the fifth population of each of the two volumes were sequenced only from day 11 of the experiments (supplementary table S1, Supplementary Material online). Each colony was inoculated into 4 ml medium in a test tube and were grown until they reached an OD of ~1. Again, this was done to reduce the time these colonies can evolve and
accumulate mutations. One milliliter of the culture was centrifuged at 10,000 g for 5 minutes and the pellet was used for DNA extraction. The remainder of each culture was then archived by freezing in 50% of glycerol in a −80°C freezer. DNA was extracted using the Qiagen DNeasy Blood & Tissue Kit. Library preparation followed the protocol outlined by Baym et al. (2015). Sequencing was carried out at the Technion Genome Center using an Illumina HiSeq 2500 machine. Clones extracted from each time point were sequenced using paired-end 150 bp reads. The ancestral clones, which were used to initiate all populations, were sequenced as well.

Calling of Mutations

To call mutations, the reads obtained for each LTSP clone or ancestral strain were aligned to the E. coli K12 MG1655 reference genome (accession NC_000913). LTSP clone mutations were then recorded in case they appeared within an LTSP clone’s genome, but not within the ancestral genome. 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).

Calculating the Proportion of Nonsynonymous versus Synonymous Mutations Expected under Neutrality

The DNA sequences of all protein-coding genes in E. coli strain MG1655 were downloaded from the NCBI database. For each position of each gene, we examined the likelihood that a mutation at this position would lead to a nonsynonymous or a synonymous change. For example, any change to the third codon position of a 4-fold degenerate codon would be synonymous, so mutations at such a position would be 100% likely to be synonymous. 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-thirds of possible mutations. In such a manner, we could add up the likelihood of a mutation being synonymous or nonsynonymous across the entirety of E. coli K12. MG1655’s protein-coding genes.

Exponential Growth Rate Estimation

To calculate the exponential growth rate of a specific LTSP clone, single colonies were used to inoculate test tubes with 4 ml of fresh LB. These cultures were grown overnight, and in the morning, they were diluted to an OD of ~0.2. Then the cultures were allowed to grow for an additional hour and...
diluted again to an OD of ~0.2 and transferred to a 96-well plate. This plate was incubated, and OD (600 nm) was read by a Neotec Infinite Pro F200 plate reader every 10 min. The exponential growth rate was calculated from the slope of the growth at the exponential phase. We used the maximum rate looking at seven consecutive time points, where $R^2 > 0.99$. For each clone, we tested two colonies with two technical replicates.

**Results**

**Culture Volume Affects Survival under LTSP**

To examine how culture volume affects the dynamics of adaptation under LTSP, we carried out LTSP evolutionary experiments, as we had previously done at 400 ml, but using culture volumes of 400, 40, and 4 ml. For each of the volumes, five independent populations were established. All the populations were grown in flasks that can contain five times the LB volume used (two-liter flasks for 400 ml experiments, 200 ml flasks for 40 ml experiments, and 20 ml flasks for 4 ml experiments). To initiate each of the ten new populations, we inoculated $\approx 10^6$ cells per ml of the *E. coli* lab strain, K12 MG1655, into the relevant LB volume. From each population, across all experiments, a sample was drawn at first daily, then weekly and eventually monthly, up to day 64. No new resources were added to the cultures throughout the experiment besides distilled water to counteract evaporation (see Materials and Methods section). We quantified the number of viable cells at each sampling time point, by plating appropriate dilutions onto LB plates and counting the resulting colonies (fig. 1A). The remainder of each sample was frozen.

As shown in figure 1A, across all the experiments, bacteria reached approximately the same maximal population size per ml of LB over the first 24 h of the experiment. However, the later decline in viability during death phase was more rapid at higher, compared with lower volumes and lower volume populations maintained higher viability upon entry into, and during LTSP. In addition to measuring colony forming units (CFUs) at each sampling point, we also measured pH (fig. 1B). Although the alkaline peak (at 24 h) of all groups was similar (~8.6), the reduction in pH levels slowed down with volume increase. These results are consistent with previous studies showing slower death within smaller volume LTSP populations and an association between alkalinization of growth media and death dynamics during death phase (Ślonczewski et al. 1981; Zilberstein et al. 1984; Booth 1985; Weiner and Model 1994; Farrell and Finkel 2003; Maurer et al. 2005; Kram and Finkel 2014).

**Lower Mutation Accumulation within Smaller Volume LTSP Populations**

For four of the five 40 ml populations and four of the five 4 ml populations, we sequenced ~10 clones per population, at each of three time points: days 11, 22, and 64. For the fifth population of each volume, we sequenced ~10 clones only from the day 11 time point (supplementary table S1, Supplementary Material online). The ancestral clones from which each population was established were also sequenced, allowing us to identify mutations that arose within each population, during its evolution under LTSP. Mutations were called using the Breseq pipeline (Deatherage and Barrick 2014). A full list of all identified mutations can be found in supplementary table S2, Supplementary Material online. For convenience, this table also contains the mutations identified in clones sequenced from these three time points from the five 400 ml populations, and already reported on in (Avrani et al. 2017).

In three of the five 400 ml populations, we found the emergence of mutators, suffering a mutation within a mismatch repair gene (Avrani et al. 2017). These mutators accumulate a higher number of mutations relative to nonmutators. No such mutators were found in any of the ten lower volume populations. Excluding any mutators found at 400 ml, we compared the numbers of mutations accumulated at days 11, 22, and 64 between populations grown at different volumes. We found that mutation accumulation increased with volume (fig. 1C).

Another way to consider the accumulation of mutations is to ask what proportion of sequenced clones maintains an entirely ancestral genotype (by acquiring no mutations under LTSP). By day 64, across all volumes, we no longer see clones that carry an entirely ancestral genotype (fig. 1D, supplementary tables S1 and S2, Supplementary Material online). Within the 400 ml populations, very few ancestral genotype clones are seen at day 11, and no such clones at all are seen by day 22. At the same time, cells carrying no mutations are able to survive at observable frequencies for longer periods of time within the lower volume LTSP populations (fig. 1D). Ancestral genotype clones are maintained at higher frequencies at days 11 and 22 within the 4 ml, compared with the 40 ml populations.

**The Majority of Mutations Accumulated across All Volume Are Likely Adaptive**

The ratio of the rates of nonsynonymous to synonymous mutations ($dN/dS$) is significantly higher than 1 for mutations acquired across all examined volumes (for the 400 ml populations we cannot calculate $dN/dS$ as there are no synonymous mutations at all and so $dS = 0$, table 1). The enrichment in nonsynonymous substitutions relative to random expectations is statistically significant for all volumes ($P \ll 0.001$, according to a $\chi^2$ test, table 1; Materials and Methods). Such an enrichment in nonsynonymous substitutions is considered to be a signal of positive selection affecting accumulated mutations (Wen-Hsiung 2002) and therefore indicates that a substantial proportion of the mutations
accumulated across volumes are likely adaptive. The highest fraction of nonsynonymous substitutions is found at 400 ml and the lowest at 4 ml. This might suggest that positive selection acts more strongly at higher compared with lower volumes. However, differences in the proportion of nonsynonymous to synonymous substitutions are only significant when comparing the 400 ml substitutions to those accumulated at 4 ml ($P = 0.04$, according to a $\chi^2$ test, table 1).

That mutation accumulation is dominated within all volume populations by positive selection is also evident from the immense convergence with which mutations accumulate across volumes (fig. 2). 30, 16, and 15 loci (genes or intergenic regions) are mutated in two or more of the five populations within 400, 40, and 4 ml, respectively. The 30 loci, which are convergently mutated across two or more of the five 400 ml populations constitute only 0.86% of the E. coli genome. Yet, 84.9% of the mutations found at days 11, 22, and 64 within these populations (excluding mutators) occur within these 30 loci. Similarly, the 16 and 15 loci convergently mutated within the 40 and 4 ml populations constitute only 0.54% and 0.46% of the E. coli genome, respectively. However, 80.5% of mutations found within the 40 ml populations and 75% of mutations found within the 4 ml populations occur within these convergently mutated loci. Such enrichment in mutations occurring within the same loci across independent populations constitutes a strong signal of positive selection. The fact that the majority of mutations found within clones evolving under LTSP occur within convergently mutated loci further indicates that the majority of mutations accumulated under LTSP are likely adaptive.

Although most mutations tend to fall within convergently mutated loci across all volumes, this trend appears to be more pronounced within the 400 ml populations. In other words, mutations occurring under LTSP at 400 ml tend to more often occur within loci that are mutated across a larger number of independent populations ($P < 0.001$ for comparisons against both 4 and 40 ml, according to a $\chi^2$ test, fig. 2). Although a higher fraction of the mutations falling within the 40 ml populations falls within convergently mutated genes (80.5%) compared with mutations falling at 4 ml (75%), this difference is not statistically significant ($P = 0.1$). Combined, these results suggest that whereas positive selection affects a majority of LTSP mutations across volumes, a higher fraction of mutations accumulated at larger volumes are likely adaptive.

Similar Genes Are Often Involved in Adaptation under LTSP across Volumes, Yet Specific Adaptive Mutations Can Vary according to Volume

Although some loci are mutated in a convergent manner in only one volume, there is substantial overlap in the identity of the loci which are convergently mutated across populations, across the different volumes (fig. 3). This suggests that similar genes are often involved in adaptation to LTSP across volumes. For example, across the three volumes, the genes rpoB and rpoC encoding the RNAPC are mutated in multiple populations. Similarly, the RNA chaperone gene proQ (Holmqvist et al. 2018), the proline metabolism genes putA (Zhang et al. 2004) and putP (Sasaki et al. 2017) and the serine/threonine transporter gene sstT (Kim et al. 2002) are all mutated convergently, across all three volumes. In addition,
multiple 400 and 40 ml populations carry mutations within the short-chain fatty acid metabolism regulator genes \( \text{fadR} \) (Campbell and Cronan 2001) and \( \text{atoS} \) (Lioliou et al. 2005), the \( \text{paaX} \) (Ferrández et al. 2000) transcriptional regulatory gene, the protease gene \( \text{clpA} \) (Gottesman et al. 1990) and within the promoter region of the \( \text{cycA} \) (Butland et al. 2005) transporter gene. On the whole, more convergently mutated genes are shared between the 40 and 400 ml populations than between either of these volume populations and the 4 ml populations. This could suggest that selective pressures might be more similar between 400 and 40 ml, but it could also be influenced by the fact that the 4 ml populations accumulated the lowest numbers of mutations overall (fig. 1C).

Mutations within the RNAPC genes \( \text{rpoB} \) and \( \text{rpoC} \) constituted the most striking example of convergent adaptation observed at 400 ml. In our previous study we reported that over 90% of 400 ml LTSP clones carried mutations within the RNAPC. We further reported that across all five 400 ml populations these mutations most frequently fell within only three specific sites of the RNAPC. Intriguingly, we find that at 40 ml there is also a very strong tendency for mutations to occur within specific sites of RpoB and RpoC proteins, across independently evolving populations. However, the identity of the protein sites at which mutations occur in a convergent manner is different between the 40 ml populations and the 400 ml populations (fig. 4). These results suggest that whereas mutations within the RNAPC are involved in adaptation to LTSP across volumes, culture volume may affect the identity of the specific sites of the RNAPC which are involved in this adaptation.

Although clones that do not carry an RNAPC adaptation appear to be quite rare within the 400 ml populations, they are much more frequent within the 40 ml populations. This corresponds well to our result presented in figure 1D, showing that as culture volume goes down, more cells are able to survive without acquiring mutations. In other words, whereas...
the patterns of high convergence in RNAPC mutations suggest that these mutations are important for LTSP adaptation at both 40 and 400 ml, it appears that more cells are able to manage for longer, under LTSP, without these adaptations at the lower culture volumes (fig. 4).

**Discussion**

Our results show that culture volume affects mutation accumulation under LTSP. Lower mutation accumulation within smaller volume LTSP populations may stem from differences in population sizes between the volumes. During the first day of growth under LTSP, across all volumes, cell numbers increase to $\sim 10^{10}$ cells per ml of culture. This means that the overall number of cells present within the 400 ml populations is about ten times higher than for the 40 ml populations and about one hundred times higher than for the 4 ml populations. This might affect the number of mutations that occur within each population by that time point, which could affect the extent of standing genetic variation available for the initial adaptation. However, the fact that mutations accumulate with time under LTSP suggests that not all genetic adaptation arises from this initial standing variation. Instead, it appears that mutations continue to occur under LTSP. At later time points, the differences in population sizes between the different volumes reduce due to the fact that at lower volumes, larger numbers of cells per ml are present within the populations (fig. 1A).

A second possible, nonmutually exclusive, explanation for differences in mutation accumulation between the various volume populations relates to the intensity of natural selection in favor of acquiring adaptations. It is quite likely that when a smaller fraction of cells survives to enter and persist under LTSP, competition between cells for survival may be stronger. As a result, it is possible that selection to carry an adaptation enabling better survival and/or growth under LTSP may be stronger under conditions imposing a stronger constraint on the number of surviving cells. Fitting with this, we observed that within smaller volume populations, that maintain higher viability (fig. 1A), a higher fraction of clones persists longer, without the acquisition of any mutations (fig. 1D).

Patterns of mutation accumulation at different volumes also suggest that positive selection may be stronger at larger volumes. Although we show that across all volumes, there is a significant enrichment in nonsynonymous versus synonymous substitutions, relative to neutral expectations, this enrichment seems to be strongest at 400 ml and weakest at 4 ml (table 1). In addition, whereas across all volumes a majority of mutations tend to fall within convergently mutated loci, this trend is again strongest at 400 ml and weakest at 4 ml (fig. 2).
populations. Mutators often emerge during evolutionary experiments and were also observed within natural bacterial populations and clinical antibiotic resistant isolates (Gross and Siegel 1981; LeClerc et al. 1996; Sniegowski et al. 1997; Giraud 2001; Voordekers et al. 2015; Mehta et al. 2019). It is thought that mutators increase in frequencies due to indirect selection in favor of linked adaptations that occur more rapidly when overall mutation rates are higher (Wielgoss et al. 2013; Good and Desai 2016; Raynes et al. 2018). Because mutators also suffer a cost due to the more rapid occurrence of deleterious mutations, mutators are thought to increase in frequency more under conditions in which there is a strong pressure to adapt (Wielgoss et al. 2013; Good and Desai 2016; Raynes et al. 2018). The fact that we observe mutators only at 400 ml and not at 40 or 4 ml may, therefore, indicate once more that the need to adapt is stronger at higher volumes.

There is substantial overlap between volumes in the identity of the loci, which are mutated in a convergent manner. At the same time, there are also examples of loci that are mutated in a convergent manner at one volume, but not within others. More overlap is seen between the convergently mutated loci found at 400 and 40 ml, compared with the overlap observed between either 400 and 40 ml and the 4 ml populations (fig. 3). This may partially stem from the occurrence of fewer mutations overall within the 4 ml populations. However, such lower mutation accumulation within the 4 ml populations cannot explain this entire pattern. For example, mutations within the genes csfC and yobF are found in four of the five 4 ml populations but do not occur in a convergent manner at 40 or 400 ml. Our results, therefore, suggest that whereas many of the same loci may be involved in adaptation to LTSP across volumes, other loci may be involved in adaptation only within specific volume LTSP populations.

The most striking example of convergent adaptation we observe involves mutations falling within the rpoB and rpoC genes that encode the RNAPC. Mutations within rpoB and rpoC are observed in all 400 and 40 ml LTSP populations, and in three of the five 4 ml LTSP populations. The majority of the RNAPC mutations occurring within the 400 ml populations fall within one of only four specific sites of the enzyme complex (fig. 4). Three of these sites are mutated across all five 400 ml LTSP populations. The remaining site is mutated in four of the five populations. Such extreme convergence strongly suggests that mutations within these specific sites of the RNAPC are likely to be strongly adaptive within the 400 ml LTSP populations. A similar trend is observed at 40 ml. The majority of RNAPC adaptations occurring within the 40 ml LTSP populations occur within one of only four specific sites of the enzyme complex. Clones mutated within one of these sites tend to appear in multiple independently evolving 40 ml LTSP populations. Again, this suggests that these four sites of the RNAPC are adaptive within the 40 ml populations. However, intriguingly the four sites of the RNAPC that are convergently mutated at 400 ml differ from those convergently mutated at 40 ml (fig. 4). It, therefore, seems that whereas the RNAPC is involved in adaptation to LTSP under all volumes, different sites of the enzyme complex may be involved in adaptation at 400 ml compared with 40 ml.

The RNAPC is responsible for all cellular transcription. Therefore, it seems reasonable to propose that adaptations within the RNAPC are likely to affect gene expression. The RNAPC emerges as a target for adaptation in numerous evolutionary experiments, in which E. coli was exposed to such diverse selective pressures as heat shock (Tenaillon et al. 2012), carbon starvation (LaCroix et al. 2015), antibiotic exposure (Degen et al. 2014), heavy metal exposure (Graves et al. 2015), and resource exhaustion (Avrani et al. 2017; Chib et al. 2017; Nandy et al. 2020). Different sites of the enzyme complex appear to be involved in adaptation to the various selective pressures. We show here that the sites of the RNAPC involved in adaptation can vary even between very similar experiments, in which only a single variable (growth volume) is altered. Why would different sites of the RNAPC tend to be involved in adaptation to different conditions, including conditions that are quite similar? Mutations to different sites of the RNAPC are likely to affect gene expression differently and the precise changes to gene expression which are adaptive are likely to vary between selective pressures. Furthermore, in addition to varying in their adaptive effects, mutations within different sites of the RNAPC likely also vary in their unintended consequences. We have previously demonstrated that the 400 ml LTSP RNAPC adaptations are highly antagonistically pleiotropic, in that they reduce the ability of bacteria to grow within fresh LB (Avrani et al. 2017). A similar analysis we carried out on the 40 ml RNAPC adaptations, reveals that they also display similar effects of reducing the ability of the cells carrying them to grow within fresh LB (supplementary fig. S1, Supplementary Material online, Materials and Methods). Because the RNAPC is involved in the transcription of all genes, it is quite likely that a mutation to the RNAPC that benefits growth under a particular condition by changing gene expression in a certain way, may also change the expression of other genes in ways that are less desirable. This likely explains these mutations’ antagonistically pleiotropic effects. In order for changes to a specific site of the RNAPC to be adaptive under a specific condition, such undesirable pleiotropic effects need to minimally manifest under that condition. It is thus possible that specific sites are involved in adaptation under specific conditions, because mutations within them change gene expression in the desired manner while minimizing adverse pleiotropic effects on gene expression. The fact that a relatively minor change in conditions leads to adaptation through such different mutations to the RNAPC may suggest that RNAPC adaptations are extremely sensitive to the conditions imposed. It is possible that under more complex conditions, such as those imposed in more
natural environments, adaptations through changes to the RNAPC would not be commonly possible.

Combined, our results demonstrate that despite the fact that we observe very convergent dynamics of adaptation under LTSP, it is possible to substantially alter these dynamics by manipulating just a single parameter of the experiment. Certain themes remain consistent across volumes, such as adaptation through the accumulation of genetic alterations, strong positive selection, highly convergent adaptation, and the identity of some of the loci at which adaptation occurs. However, we also observe substantial differences, in the rates of mutation accumulation, in the intensity of positive selection affecting mutation accumulation, and in the adaptations themselves. The effects of altering a single parameter of our evolutionary experiment are likely not unique to this evolutionary experimental setup. By varying parameters of other evolutionary experiments, we should also be able to understand which elements of the experiment can be easily manipulated and which remain stable to minor perturbations.

**Supplementary Material**

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

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**Data availability**

Data of all mutations accumulated within the analyzed LTSP populations, at the analyzed time points are provided in supplementary table S2, Supplementary Material online. The raw sequence data have been deposited in the Sequence Read Archive (SRA) under BioProject PRJNA669353.

**Literature Cited**

Avrani S, Bolotin E, Katz S, Hershberg R. 2017. Rapid genetic adaptation during the first four months of survival under resource exhaustion. Mol Biol Evol. 34(4):1758–1769.

Battesti A, Majdalani N, Gottesman S. 2011. The RpoS-mediated general stress response in Escherichia coli. Annu Rev Microbiol. 65(1):189–213.

Baym M, et al. 2015. Inexpensive multiplexed library preparation for megabase-sized genomes. PLoS One 10(5):e0128036.

Booth R. 1985. Regulation of cytoplasmic pH in bacteria. Microbiol Rev. 49(4):359–378.

Butlant G, et al. 2005. Interaction network containing conserved and essential protein complexes in Escherichia coli. Nature 433(7025):531–537.

Campbell JW, Cronan JE. 2001. Escherichia coli FadR positively regulates transcription of the fabB fatty acid biosynthetic gene. J Bacteriol. 183(20):5982–5990.

Chib S, Ali F, Seshasayee ASN. 2017. Genomewide mutational diversity in Escherichia coli population evolving in prolonged stationary phase. mSphere 2(3):1–15.

Deatherage DE, Barrick JE. 2014. Identification of mutations in laboratory-evolved microbes from next-generation sequencing data using Breseq. In: Sun L, Shou W, editors. Engineering and Analyzing Multicellular Systems. Vol. 1151. New York: Springer. p. 165–188.

Degen D, et al. 2014. Transcription inhibition by the depsipeptide antibiotic salinamide A. elife 3:1–29.

Farrell MJ, Finkel SE. 2003. The growth advantage in stationary-phase phenotype conferred by rpoS mutations is dependent on the pH and nutrient environment. JB 185(24):7044–7052.

Ferrández A, García JL, Díaz E. 2000. Transcriptional regulation of divergent paa catabolic operons for phenylacetic acid degradation in Escherichia coli. J Biol Chem. 275(16):12214–12222.

Finkel SE. 2006. Long-term survival during stationary phase: evolution and the GASP phenotype. Nat Rev Microbiol. 4(2):113–120.

Finkel SE, Kolter R. 1999. Evolution of microbial diversity during prolonged starvation. Proc Natl Acad Sci. 96(7):4023–4027.

Giraud A. 2001. Costs and benefits of high mutation rates: adaptive evolution of bacteria in the mouse gut. Science (80-) 291(5513):2606–2608.

Good BH, Desai MM. 2016. Evolution of mutation rates in rapidly adapting asexual populations. Genetics 204(3):1249–1266.

Gottesman S, Clark WP, Maurizi MR. 1990. The ATP-dependent Clp protease of Escherichia coli. sequence of clpA and identification of a Clp-specific substrate. J Biol Chem. 265(14):7886–7893.

Graves JL, et al. 2015. Rapid evolution of silver nanoparticle resistance in Escherichia coli. Front Genet. 6:1–13.

Gross MD, Siegel EC. 1981. Incidence of mutator strains in Escherichia coli and coliforms in nature. Mutat Res Lett. 91(2):107–110.

Hofmeyr E, Li L, Bischler T, Barquist L, Vogel J. 2018. Global maps of ProQ binding in vivo reveal target recognition via RNA structure and stability control at mRNA 3’ ends. Mol Cell 70(5):971–982.e6.

Kim Y-M, et al. 2002. Purification, reconstitution, and characterization of Na+/Asp symporter, SatT, of Escherichia coli. J Biochem. 132(1):71–76.

Kram KE, Finkel SE. 2014. Culture volume and vessel affect long-term survival, mutation frequency, and oxidative stress of Escherichia coli. Appl Environ Microbiol. 80(5):1732–1738.

Kram KE, Finkel SE. 2015. Rich medium composition affects Escherichia coli survival, glycation, and mutation frequency during long-term batch culture. Appl Environ Microbiol. 81(13):4442–4450.

Kram KE, et al. 2017. Adaptation of Escherichia coli to long-term serial passage in complex medium: evidence of parallel evolution. mSystems 2(2):1–12.

LaCroix RA, et al. 2015. Use of adaptive laboratory evolution to discover key mutations enabling rapid growth of Escherichia coli K-12 MG1655 on glucose minimal medium. Appl Environ Microbiol. 81(1):17–30.

LeClerc JE, Li B, Payne WL, Cebula TA. 1996. High mutation frequencies among Escherichia coli and salmonella pathogens. Science (80-) 274(5290):1208–1211.

Liao C-H, Shellenberger LM. 2003. Survivability and long-term preservation of bacteria in water and in phosphate-buffered saline. Lett Appl Microbiol. 37(1):45–50.

Liolou EE, et al. 2005. Phosphorylation activity of the response regulator of the two-component signal transduction system AtoS–AtoC in E. coli. Biochim Biophys Acta 1725(3):257–268.

Maurer LM, Yohannes E, Bondurant SS, Radmacher M, Slonczewski JL. 2005. pH regulates genes for flagellar motility, catabolism, and oxidative stress in Escherichia coli K-12. JB 187(1):304–319.
Mehta HH, et al. 2019. The essential role of hypermutation in rapid adaption to antibiotic stress. Antimicrob Agents Chemother. 63:1–18.
Nandy P, Chib S, Seshasayee A. 2020. A mutant RNA polymerase activates the general stress response, enabling *Escherichia coli* adaptation to late prolonged stationary phase. mSphere 5(2):1–16.
Raynes Y, Wylie CS, Sniegowski PD, Weinreich DM. 2018. Sign of selection on mutation rate modifiers depends on population size. Proc Natl Acad Sci USA. 115(13):3422–3427.
Riedel TE, Berelson WM, NealsonKH, Finkel SE. 2013. Oxygen consumption rates of bacteria under nutrient-limited conditions. Appl Environ Microbiol. 79(16):4921–4931.
Rozen DE, Philippe N, Arjan de Visser J, Lenski RE, Schneider D. 2009. Death and cannibalism in a seasonal environment facilitate bacterial coexistence. Ecol Lett. 12(1):34–44.
Sasaki H, Sato D, Oshima A. 2017. Importance of the high-expression of proline transporter PutP to the adaptation of *Escherichia coli* to high salinity. Biocntrol Sci. 22(2):121–124.
Slonczewski JL, Rosen BP, Alger JR, Macnab RM. 1981. pH homeostasis in *Escherichia coli*: measurement by 31P nuclear magnetic resonance of methylphosphonate and phosphate. Proc Natl Acad Sci. 78(10):6271–6275.
Sniegowski PD, Gerrish PJ, Lenski RE. 1997. Evolution of high mutation rates in experimental populations of *E. coli*. Nature 387(6634):703–705.
Tenillon O, et al. 2012. The molecular diversity of adaptive convergence. Science (80-) 335(6067):457–461.
Voordecker K, et al. 2015. Adaptation to high ethanol reveals complex evolutionary pathways. PLoS Genet. 11(11):e1005635.
Weiner L, Model P. 1994. Role of an *Escherichia coli* stress-response operon in stationary-phase survival. Proc Natl Acad Sci. 91(6):2191–2195.
Wen-Hsiung LG. 2002. Fundamentals of molecular evolution. 2nd edition. Q Rev Biol. 77:57.
Wielgoss S, et al. 2013. Mutation rate dynamics in a bacterial population reflect tension between adaptation and genetic load. Proc Natl Acad Sci. 110(1):222–227.
Zambrano MM, Kolter R. 1993. *Escherichia coli* mutants lacking NADH dehydrogenase I have a competitive disadvantage in stationary phase. J Bacteriol. 175(17):5642–5647.
Zambrano MM, et al. 1993. Microbial competition: *Escherichia coli* mutants that take over stationary phase cultures. Science (80-) 259(5102):1757–1760.
Zhang M, et al. 2004. Structures of the *Escherichia coli* PutA proline dehydrogenase domain in complex with competitive inhibitors †, ‡. Biochemistry 43(39):12539–12548.
Zilberstein D, Agmon V, Schuldiner S, Pidan E. 1984. *Escherichia coli* intracellular pH, membrane potential, and cell growth. J Bacteriol. 158(1):246–252.

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