Evolution Letters, 2023, 7(4), 252–261
https://doi.org/10.1093/evlett/qrad019
Letter

Hypermutator emergence in experimental *Escherichia coli* populations is stress-type dependent

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Abstract

Genotypes exhibiting an increased mutation rate, called hypermutators, can propagate in microbial populations because they can have an advantage due to the higher supply of beneficial mutations needed for adaptation. Although this is a frequently observed phenomenon in natural and laboratory populations, little is known about the influence of parameters such as the degree of maladaptation, stress intensity, and the genetic architecture for adaptation on the emergence of hypermutators. To address this knowledge gap, we measured the emergence of hypermutators over ~1,000 generations in experimental *Escherichia coli* populations exposed to different levels of osmotic or antibiotic stress. Our stress types were chosen based on the assumption that the genetic architecture for adaptation differs between them. Indeed, we show that the size of the genetic basis for adaptation is larger for osmotic stress compared to antibiotic stress. During our experiment, we observed an increased emergence of hypermutators in populations exposed to osmotic stress but not in those exposed to antibiotic stress, indicating that hypermutator emergence rates are stress type dependent. These results support our hypothesis that hypermutator emergence is linked to the size of the genetic basis for adaptation. In addition, we identified other parameters that covaried with stress type (stress level and IS transposition rates) that might have contributed to an increased hypermutator provision and selection. Our results provide a first comparison of hypermutator emergence rates under varying stress conditions and point towards complex interactions of multiple stress-related factors on the evolution of mutation rates.

Keywords: hypermutator, experimental evolution, antibiotics, osmotic stress, insertion sequence, genetic architecture

Lay Summary

Mutation is a double-edged sword. On the one hand, a mutation can have a detrimental effect on an organism by affecting its proper functioning. On the other hand, a mutation can be beneficial when it increases adaptation to an environment. An organism is therefore expected to benefit from having a low mutation rate in an environment to which it is already well adapted. However, when an organism reproduces in an environment to which it is not well adapted, a higher mutation rate can be beneficial as it increases the chances of producing offspring that have an adaptive mutation. Hypermutators are bacteria with a genetically determined high mutation rate. These hypermutators have often been observed in populations that are adapting to stressful environments, but it is currently not well understood which specific conditions lead to their evolution. In this paper, we followed the evolution of hypermutators in *Escherichia coli* populations grown in presence of different types and levels of stress. Our results point towards the importance of the number of genes involved in adaptation to a particular stress: the higher the number of genes that contribute to adaptation to a stress, the higher the chances that hypermutators evolve under those stress conditions. But the story is not that simple: we also discovered that certain types of stress cause some mobile genetic elements to jump around more in the genome, and this might also contribute to the evolution of hypermutators.

Introduction

Hypermutators are bacterial isolates exhibiting elevated genome-wide mutation rates and are frequently observed in both experimental and natural populations (e.g., Denamur et al., 2002; LeClerc et al., 1996; Oliver and Mena, 2010; Sniegowski et al., 1997). The genetic determinant of hypermutability is often a loss-of-function mutation in one of the genes for the methyl-directed mismatch repair (MMR) system. This type of mutation causes an increase in point mutation rates, a shift in mutation bias, and an enhanced integration of foreign genetic material (Horst et al., 1999; Lee et al., 2012; Rayssiguier et al., 1989). These hypermutator alleles are by themselves often not adaptive [although see e.g., Torres-Barceló et al. (2013) for potential direct benefits of defunct MMR genes] and they can even be deleterious by causing an increased mutational load and higher mortality (Swings et al., 2017; Tröbner & Piechocki, 1984). However, hypermutator alleles can experience indirect positive selection when they hitchhike with linked adaptive mutations, which they generate at a higher

Received February 9, 2023; revisions received April 4, 2023; accepted April 21, 2023

rate than normomutators. Theoretical models have shown that this indirect positive selection allows them to propagate and fix within asexual populations (Taddei et al., 1997; Tenaillon et al., 1999). Additionally, shifts in mutation bias can result in a different distribution of fitness effects (DFE) of the generated mutations and further prompts indirect selection and adaptation by different evolutionary paths (Couce et al., 2013).

Several factors are known to influence the propagation and fixation of hypermutator alleles in bacterial populations. Early experiments indicated that there is frequency-dependent selection on hypermutators, with fixation of hypermutator alleles only occurring when the initial frequency within a population is above a certain threshold (Chao & Cox, 1983). The proposed explanation was that when hypermutators compete with a much larger population of normomutators, the overall number of mutations (and thus the likelihood of generating an adaptive mutation) will still be higher in the normomutator population. However, this view was recently challenged by Raynes and Weinreich (2019) who demonstrated that although the fixation probability of a hypermutator increases with its frequency, the per-capita fixation probability of hypermutators is independent of their frequency. This allows for the possibility that hypermutators that are initially present at a very low frequency could rise and fix in a population (although rarely), in line with the experimental observation of spontaneous hypermutator emergence in large normomutator populations (Pal et al., 2007; Sniegowski et al., 1997; Swings et al., 2017). Population size also influences indirect selection on hypermutators in closed populations: hypermutators have been shown to be favored in large populations but suppressed in small populations (Raynes et al., 2018, 2019).

The indirect advantage of hypermutators has been predicted to be stronger when a population is far from its fitness optimum, i.e., when it is in environmental conditions to which it is not well adapted (Tenaillon et al. 1999). Indeed, when a population is far from its optimum the DFE of mutations is broader with a larger proportion of beneficial mutations (Hietpas et al., 2013; Martin & Lenormand, 2015). Nonoptimal conditions thus increase the availability of beneficial mutations for hypermutators to generate and hitchhike on. Some experimental results are in line with this prediction: hypermutators are more likely to emerge and propagate in *Pseudomonas fluorescens* populations that coevolve with an infecting phage compared to populations evolving in the absence of this phage (Pal et al., 2007). The same study also showed that hypermutators have a competitive advantage over normomutators in the presence of this phage, but not in the absence of it. Other studies also noted a higher occurrence of hypermutators in experimental populations adapting to antibiotic stress (Hammerstrom et al., 2015; Mao et al., 1997). Finally, Swings et al. (2017) showed that the occurrence of hypermutators allows *Escherichia coli* populations to adapt to higher concentrations of ethanol compared to populations where no hypermutators appeared.

The advantage of hypermutators when adapting to a new environment can, however, be dependent on the actual mutation rate. Sprouffske et al. (2018) showed *E. coli* lines with a moderate increase in mutation rate adapt faster to a stressful environment than lines with the highest mutation rate. Additionally, a reduction in the mutation rate of hypermutators has been documented in evolution experiments that were initiated with hypermutator lines (Ho et al., 2021) or where some populations became hypermutator (Swings et al., 2017; Wielgoss et al., 2013). This reduction in mutation rate was likely selected when populations adapted and moved closer to their optimum, causing the fraction of advantageous mutations and the indirect fitness advantage of hypermutators to diminish, and deleterious mutations to accumulate.

It is clear from the examples above that hypermutator emergence is strongly influenced by selective conditions. However, to our knowledge rates of hypermutator emergence in different stressful conditions have not been compared. It is difficult to derive this by comparing earlier studies, because these studies do not only differ in selective conditions but also in the length of the experiment, genetic background of the bacterial strain, and population dynamics. We addressed this knowledge gap by exposing experimental *E. coli* populations to three levels of either antibiotic stress (the aminoglycoside gentamicin) or osmotic stress (elevated salinity) for approximately 1,000 generations. These two stress types were chosen because the number of potential beneficial mutations is expected to differ between them. Aminoglycoside antibiotic stress is highly specific through inhibition of protein synthesis by binding to the 30S ribosomal subunit and can therefore be considered as a specific stress with a limited number of loci involved in adaptation (Ibacache-Quiroga et al., 2018). In contrast, osmotic stress activates the global regulator σ^S that coordinates expression of up to 10% of the genome (Weber et al., 2005) and is expected to affect many processes of the cell's physiology. Osmotic stress can therefore be considered as a general stress with many loci potentially contributing to adaptation. Based on this, we tested the prediction that the DFE is more biased toward beneficial mutations when adapting to osmotic stress compared to antibiotic stress, thus favoring the emergence of hypermutators.

Methods

Escherichia coli strain

The *E. coli* strain DH10B ("Top10 competent cells," Thermo Fisher Scientific) was used in this experiment. This strain was chosen because earlier experiments showed that it is prone to the generation of hypermutator phenotypes through IS10 insertion in the *mutS* and *mutL* genes (Bedhomme et al., 2019). IS10 is furthermore known to have very high transposition rates in *E. coli* (Shen et al., 1987) and IS10 insertions are thus a common hypermutator inducing mutation in *E. coli* DH10B. This characteristic allowed us to obtain a fine-grained picture on the emergence of hypermutators throughout experimental evolution using a PCR screening assay for IS10 insertions in *mutS* and *mutL* (see below for details on the screening assay).

Experimental evolution

Escherichia coli DH10B populations were exposed to either antibiotic stress (gentamicin) or osmotic stress (elevated NaCl concentration) for ~1,000 generations. For each stress type, 12 replicate *E. coli* populations were exposed to each of three stress levels that reduced the OD_{24h} by either 20%, 40%, or 60% relative to populations growing in standard LB (Supplementary Figure S1). Prior to determining dose–response curves for osmotic stress, *E. coli* was exposed to the respective salt concentrations for four serial passages because of a strong initial acclimation to the salt medium. During this period, the dose–response curve showed strong shifts after each passage and we hypothesized this was due to phenotypic plasticity rather than an evolutionary response. Such acclimation was not observed for antibiotic stress, so dose–response curves were determined in the first passage for this stressor. Due to technical limitations, we were not

able to define our stress levels based on a reduction in maximum population growth rate (μ_{max}), but for both stress types there was a good correlation between μ_{max} and OD_{max} (Supplementary Figure S2), so we consider stress intensity evaluated on OD_{24h} as representative of stress intensity in terms of growth rate reduction. For the antibiotic stress, the concentrations of gentamicin corresponding to the three stress levels were 0.5, 1.0, and 2.0 µg/mL. For osmotic stress, these stress levels were obtained by adding respectively 0.75 M (43.8 mg/mL), 0.80 M (46.8 mg/mL), and 0.85 M (49.7 mg/mL) of NaCl to standard LB broth (already containing 5 mg/mL of NaCl). As a control treatment, 24 populations were propagated in standard LB medium, resulting in a total of 96 populations.

All 96 populations were established by inoculating 10 µL of an overnight culture grown from a single colony of *E. coli* DH10B (the *ancestral* population). Populations were maintained in 1 mL of the appropriate medium on two 96-well plates (Greiner deepwell). Experimental populations were organized in a checkerboard pattern on the plates, with sterile LB medium in alternating wells to identify any cross-contamination (Supplementary Figure S3). Populations were grown at 37°C and 300 rpm orbital shaking. Every day, 10 µL of each population was transferred to a new plate containing fresh medium (10^{-2} dilution, giving approximately 6.6 generations/day). Populations were maintained for 148 days, resulting in approximately 1,000 generations of experimental evolution (these final populations are referred to as the *evolved* populations). Frozen archives of the evolving populations were stored twice a week in the form of glycerol stocks. After 1,000 generations, one population exposed to the highest level of osmotic stress was found to be contaminated by non-*E.coli* bacteria and was excluded from further analysis.

Detection of hypermutator emergence during experimental evolution

As a previous experiment had shown that in *E. coli* DH10B hypermutators were often induced through IS10 insertions in either the *mutL* or *mutS* genes (Bedhomme et al., 2019), we screened all populations during experimental evolution for this type of mutation. The presence of IS10-induced hypermutators was checked every two weeks using a PCR assay. The *mutS* and *mutL* genes were amplified with primers binding before the start codon and after the stop codon in all experimental populations, and the presence of an IS10-triggered hypermutator and its potential fixation in the population were determined from amplicon size on an agarose gel (see Supplementary Table S1 for primer sequences and PCR conditions). Whenever the emergence of a hypermutator was detected in a population, the three glycerol stocks stored since the previous PCR assay were additionally screened to determine the time of emergence more precisely (resulting in a precision of approximately 20 generations). The effect of the type and level of stress on differences in emergence rates of IS10-induced hypermutators between treatments were analyzed by survival analysis on right-censored survival curves with a log-rank test using the "survival" package in R (Therneau, 2021).

Quantification of adaptation

We used two datasets to quantify the adaptation of the evolved populations to the stress they were exposed to. The first dataset contained OD_{24h} measurements for all populations obtained during experimental evolution on every third passage prior to transfer. The second dataset contained growth kinetic parameters of ancestral and evolved populations measured after experimental evolution.

To quantify adaptation based on OD_{24h} measurements taken during experimental evolution, OD_{24h} values were first normalized relative to the mean OD_{24h} of all control populations on the same day. For populations evolving under osmotic stress, only OD_{24h} measurements after the fifth transfer were considered due to the strong initial acclimation (see Experimental evolution). For each population, the change in normalized OD_{24h} as a function of transfer number was then quantified by fitting a linear regression. Population adaptation was signaled when the slope of the regression was positive and significantly different from zero.

To quantify adaptation based on growth kinetic parameters, liquid cultures of the ancestral and evolved populations were initiated from frozen glycerol stocks, and grown overnight in standard LB medium. Growth rate measurements under gentamicin exposure were performed on populations inoculated with the initial overnight culture. For growth rate measurements under salt exposure, the inoculum was first acclimatized to the salt concentration at which the growth rate would be measured by four serial passages in the respective salt concentration. Growth rates of the ancestral population were determined in standard LB and at all stressor levels ($n = 8$ for each gentamicin concentration, $n = 12$ for each salt concentration). The growth rate of each evolved population was determined in standard LB and at the stressor level it evolved in ($n = 4$ for populations evolved in gentamicin, $n = 3$ for populations evolved in salt). Growth rates were determined by measuring OD kinetics over a period of 24 hrs on populations growing in a Tecan Spark spectrophotometer (37°C, shaking every 3 min, OD_{600} measurements every 30 min). The maximal growth rate (μ_{max}) and maximal OD (OD_{max}) of each population was determined on spline fitted growth curves using the R package grofit (Kahm et al., 2010). The relative growth rate (μ_{rel}) and relative OD (OD_{rel}) of a population were determined by dividing the μ_{max} or OD_{max} when exposed to a specific stress level by the μ_{max} or OD_{max} when grown in standard LB medium. Adaptation of evolved populations was signaled when the μ_{rel} or OD_{rel} was higher than the upper limit of the 95% confidence interval for respectively the μ_{rel} and OD_{rel} of the ancestral population.

Genomic analysis of the evolved populations

To obtain the whole genome sequence of the 95 evolved populations and the ancestral population, libraries were prepared as follows: total DNA was extracted from 4 mL of overnight cultures grown from glycerol stocks of the ancestral and evolved populations using the Qiagen Blood and Tissue Kit. DNA library preparation was performed using the Illumina Nextera Flex DNA Library Kit following manufacturer instructions. Libraries were paired-end sequenced (2 × 150 bp) on a Novaseq 6000 at Genewiz (Leipzig, Germany). The fastq files were cleaned using PRINSEQ (Schmieder & Edwards, 2011): The first 11 bp of each read were removed, bases downstream of a base with a quality score $Q < 30$ were removed, reads with undetermined bases and duplicated reads were eliminated. Mapping and mutation identification were done using the BRESEQ pipeline (Deatherage & Barrick, 2014) with the polymorphism detection option, as the sequencing was done at the population level. Reads were mapped on the *E. coli* K12 DH10B genome (GenBank accession number: CP000948.1). Good quality resequencing was obtained for the 24 control populations, 35 out of the 36 populations evolved under antibiotic stress, and 33 out of the 35 populations evolved under osmotic

stress. We detected one difference between the ancestral genome and the reference sequence (one additional IS10 insertion), which was subtracted from the mutations identified in the evolved populations. The BRESEQ outputs were cleaned manually to remove false polymorphisms due to mapping errors between high homology zones. All mutations detected with a frequency above 0.05 were kept in the dataset.

UPGMA hierarchical clustering was used to determine whether mutational profiles differed between stress types and stress levels. A zero-one matrix was constructed to indicate which loci (defined as protein-coding genes and their intergenic regions) were mutated in each population. This matrix included all populations exposed to osmotic- or antibiotic stress, and loci that were mutated in at least two of these populations. Clustering was based on the Jaccard distance between populations. Populations were subsequently partitioned into four discrete clusters using k-means clustering.

IS transpositions were determined based on the population level whole genome sequencing data. Differences in IS transposition rates were determined by comparing values of populations exposed to either osmotic stress or antibiotic stress to those of control populations using a nonparametric Mann–Whitney *U*-test.

Identification of loci involved in stress adaptation

Because mutational profiles were well-differentiated between stress types but not between stress levels (see Results), adaptive mutations were identified for each stress type by considering all stress levels together for each stress type. This resulted in an increased statistical power to detect loci in which mutations were adaptive when exposed to a particular stress type.

To identify which mutations were likely to be adaptive to a specific stress, we first determined which loci (a locus being a protein coding gene or an intergenic region) were mutated in parallel among populations of the same evolutionary treatment. For all loci that were mutated in at least two populations of the same evolutionary treatment, we determined whether the number of populations mutated in parallel was greater than expected at random based on simulations. We first attributed a mutation rate to each population, calculated as the number of changes detected in this population divided by the genome length. These rates are highly heterogeneous because some populations became hypermutators during the experiment. Using the mutation rates in each population exposed to a particular stress type and the length of each locus, we simulated the evolution of each locus in each population 10^5 times. These simulations mimic what the level of parallel evolution would have been for each locus if all changes were due to mutation only (i.e., without the action of selection). Locus *L* mutated in *n* populations in the experimental data was attributed the proportion of simulations where locus *L* got mutated in at least *n* populations as the probability of getting this level of parallel mutation by chance (i.e., as *p*-value). All loci which were attributed a *p*-value below .001 were considered as being mutated in parallel significantly more than by chance, and mutations within them as being very likely adaptive. This resulted in a list of loci for populations evolved in presence of a particular stress and for populations evolved in absence of it. The loci in which mutations are likely adaptive in a particular stress were defined as loci present in the list derived from populations evolved in presence of the stress and absent from the list derived from populations evolved in absence of the stress. Loci belonging to both lists are likely to correspond to adaptation to the conditions of the experimental evolution protocol. A jackknife resampling was performed to obtain a confidence estimate on the obtained number of loci involved in adaptation for each stress type. This was done by determining the loci in which mutations are likely adaptive for an $n-1$ subsample of the populations of a particular stress type ("leave one out" analysis). The difference in the number of adaptive loci between stress types was analyzed by comparing the obtained distributions using a two-sample *t*-test.

Results

The emergence of hypermutators is elevated when exposed to osmotic stress

A higher emergence rate of hypermutators was observed in populations exposed to osmotic stress compared to populations exposed to antibiotic stress or control populations (Figure 1).

When considering the emergence of hypermutators based on the detection of IS10 insertions in the *mutS* or *mutL* genes during experimental evolution, there was an overall significant effect of stress type on the rate of emergence (log-rank $\chi^2 = 18.1$ [$df = 2$, $N = 95$], $p = .0001$), but no significant effect of stress level (log-rank $\chi^2 = 6.6$ [$df = 2$, $N = 95$], $p = .09$). In the control treatment there were no detectable IS10 insertions in *mutS* or *mutL* during experimental evolution. In populations exposed to gentamicin, an IS10 insertion was detected once in *mutL* which did not reach fixation during our experiment, and once transiently in *mutS*. In populations exposed to osmotic stress, eleven IS10 insertions were detected in *mutS*, of which six reached fixation within 1,000 generations. An IS10 insertion in *mutL* was also detected in one population exposed to osmotic stress, where it reached fixation. Contrasting the emergence of hypermutators in exposed treatments to the control treatment indicated that emergence was higher for populations exposed to osmotic stress (log-rank $\chi^2 = 9.9$ [$df = 1$, $N = 59$], $p = .009$), but not for populations exposed to antibiotic stress (log-rank $\chi^2 = 1.4$ [$df = 1$, $N = 60$], $p = .2$; Figure 1B).

The analysis of whole genome sequences of the evolved populations confirmed the hypermutators detected by the PCR assay and their fixed/polymorphic status at generation 1,000. This WGS data also revealed the presence of additional hypermutators in several populations caused by nonsynonymous mutations in one of the genes known for their potential to induce strong hypermutator phenotypes when affected (*dnaQ, mutS, mutL, mutH, uvrD, mutM, mutY*, or *mutT*; Horst et al., 1999; SupplementaryTable S2). The number of mutations in these populations at generation 1,000 was between 4.7 and 65.4 times higher than the median number of mutations in normomutator populations, confirming that they were indeed hypermutators (Supplementary Figure S4). When considering hypermutators due to both IS interruption in *mutS* and *mutL* and other nonsynonymous mutations in the above-mentioned genes, there was an overall significant effect of stress type on the rate of emergence (Pearson's $\chi^2 = 12.1$, $df = 2$, $p = .002$) but no effect of the stress level (Pearson's $\chi^2 = 6.4$, $df = 3$, $p = .09$). The number of populations containing hypermutators was not significantly different between the control and the antibiotic stress treatments ($p = .5$), while the osmotic stress treatments differed significantly from both the control ($p = .006$), and the antibiotic stress treatments ($p = .02$; Figure 1C).

More loci are implicated in adaptation to osmotic stress compared with adaptation to antibiotic stress

All populations showed signs of adaptation to the stress they were exposed to. Changes in OD_{24h} during experimental evolution were highly variable between treatments. Most populations exposed to

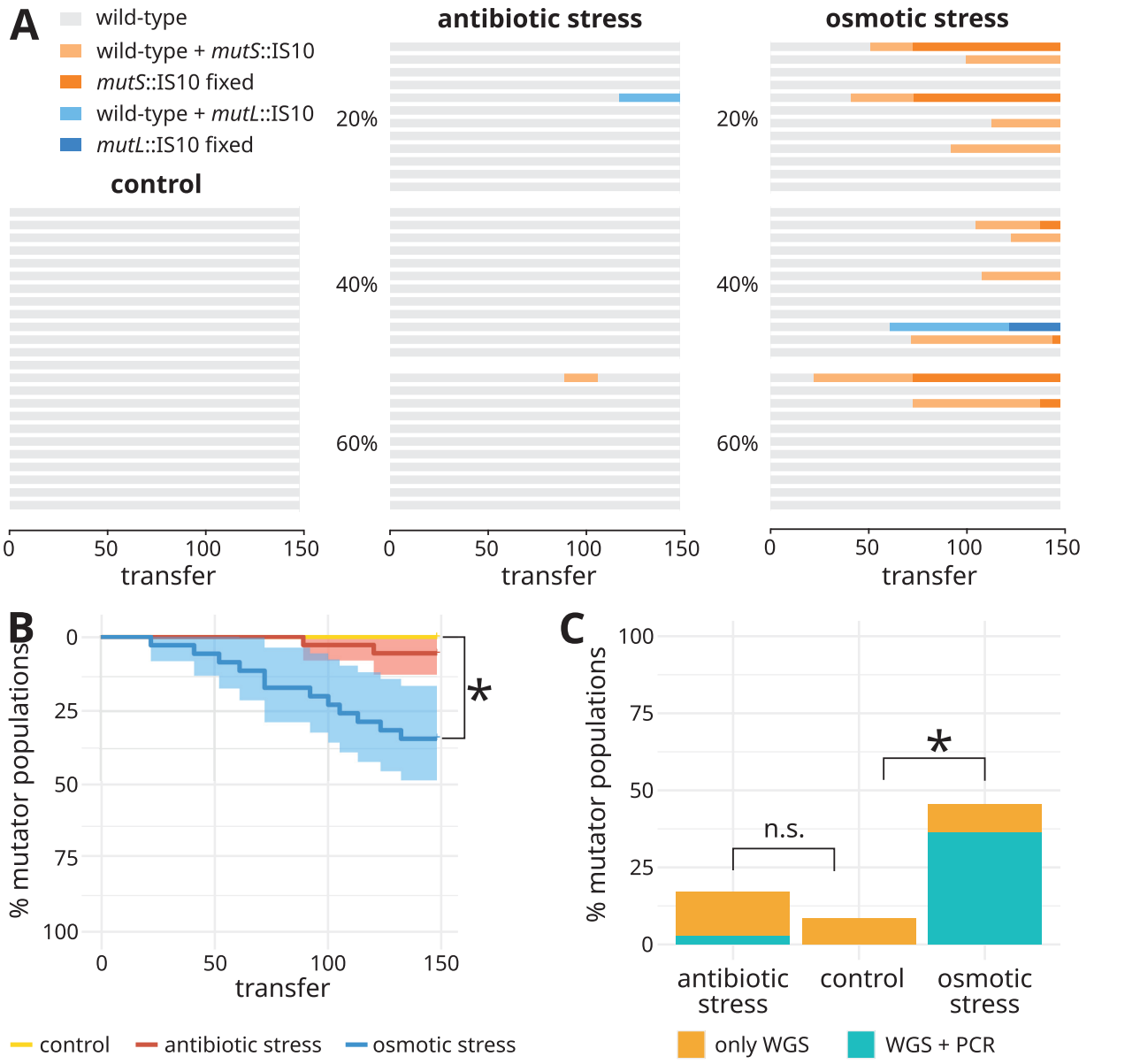

Figure 1. Emergence of hypermutators during experimental evolution. (A) Detection of IS10 insertions in mutS and *mutL* genes during experimental evolution. Each line represents a population and populations are grouped by stress type and stress level (indicated by the % reduction of OD_{24h} in the ancestor). (B) Kaplan–Meier curve with confidence intervals indicating the emergence of hypermutator genotypes over populations grouped by stress type based on the PCR assay. An asterisk indicates a significant difference in rate of emergence as determined by a log-rank test. (C) Percentage of populations determined to contain hypermutators by population level whole genome sequencing (WGS) at the end of the experimental evolution. An asterisk indicates a significant difference as determined by a Pearson's chi-squared test.

antibiotic stress showed a significant increase in OD_{24h} during the experiment (except for two populations). Higher antibiotic concentrations also generally resulted in a stronger increase in OD_{24h} (Figure 2A; mean slope A40% and A60% > mean slope A20%, Tukey HSD p-adj. < .05). In contrast, almost half of the populations (17 out of 36) evolving under exposure to osmotic stress did not show a significant increase in OD_{24h} during the experiment, with no apparent trend related to stress level. Based on population growth kinetics, all populations showed indications of adaptation, with μ_{rel} of all evolved populations being higher than μ_{rel} of the ancestor (Figure 2B). A similar pattern was observed for changes in OD_{rel}, except for two evolved populations exposed to the intermediate level of osmotic stress whose OD_{rel} was not higher than the OD_{rel} of the ancestral population (Supplementary Figure S5). We found no indications that hypermutator populations adapted better to antibiotic or osmotic stress compared to normomutator populations (two sample t-test comparing hypermutator and normomutator populations within a stress type, for all metrics $p > .05$).

Hierarchical clustering of the evolved populations based on the presence/absence of mutations in specific loci shows that, in general, populations evolved in the presence of the same stress type have more similar mutational profiles (Figure 2C). Notable exceptions are three antibiotic stress hypermutator populations that cluster together with several osmotic stress hypermutator populations (forming a hypermutator-only cluster), an antibiotic stress population clustering with osmotic stress populations, and an antibiotic stress population that does not belong to any other cluster. Interestingly, the cluster with only hypermutator populations contains those populations that accumulated the largest number of mutations by the end of the experiment (between

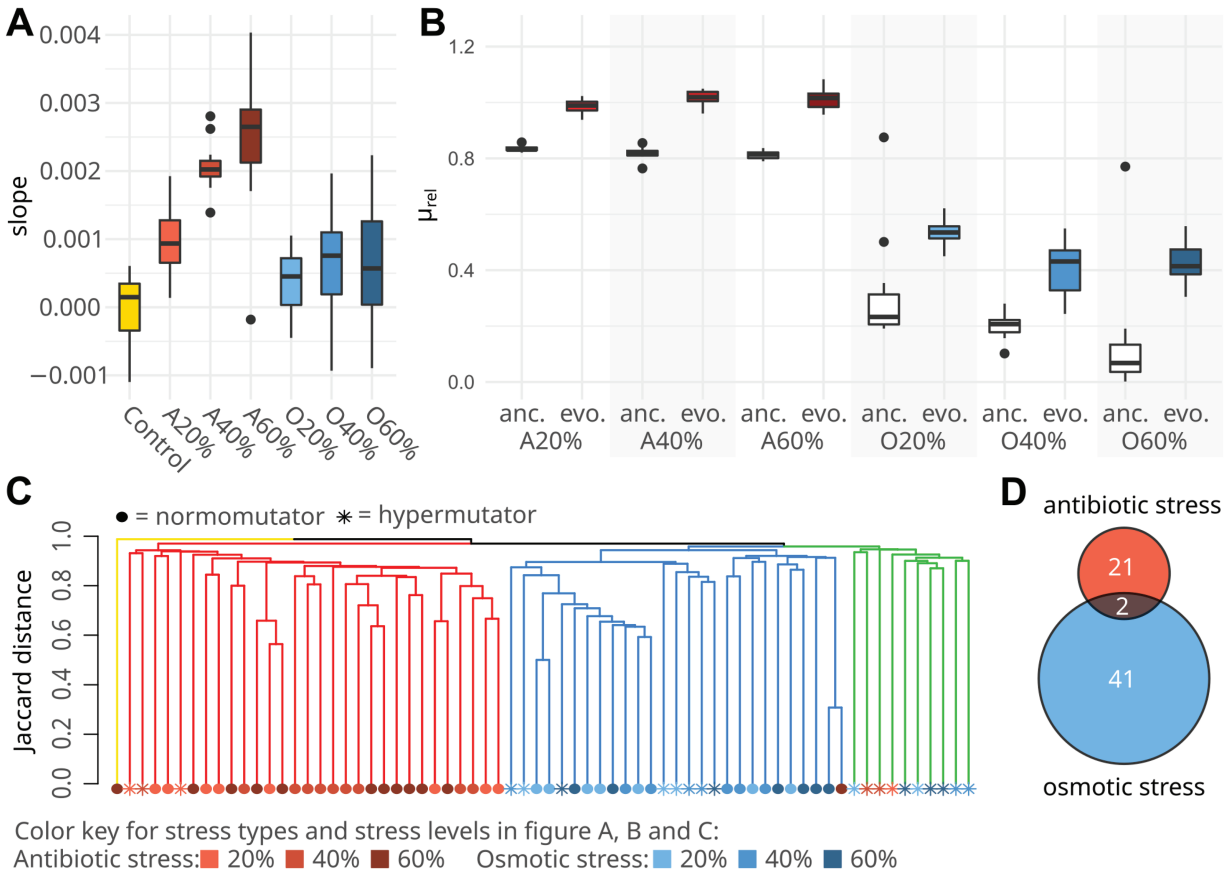

Figure 2. Evolutionary responses. (A) Slope values of a linear model fitted to OD_{24h} measurements taken throughout the experiment on evolving populations. Higher slope values indicate stronger adaptive responses. Slope estimates are grouped by stress type (A = antibiotic stress, O = osmotic stress) and stress level (indicated by the % reduction of OD_{24h} in the ancestor). The horizontal line within the boxplot indicates the median value, boxes span the 25th–75th percentile, and vertical lines the max. and min. value. Dots indicate outliers. (B) Changes in relative growth rate (μ_{rel}) after evolving in antibiotic or osmotic stress determined on population growth kinetics. For each environment μ_{rel} was determined for both the ancestral genotype ("anc."; left boxes within a treatment) and after evolution in a particular environment ("evo."; right boxes within a treatment). Boxplots same as in (A). (C) UPGMA hierarchical clustering of antibiotic stress and osmotic stress populations based on the Jaccard distance of their mutational profile. Branch colors indicate clusters identified by k-means clustering. (D) Venn diagram indicating the inferred number of loci contributing to adaptation for both stress types.

113 and 654 mutations), while hypermutator populations in other clusters accumulated fewer mutations (between 47 and 105 mutations). No apparent clustering according to stress level within stress types is discernible.

The number of loci contributing to adaptation to osmotic stress (43 loci; jackknife estimate = 41.18 ± 1.1; Figure 2D) was significantly higher than the number of loci contributing to adaptation to antibiotic stress (23 loci, jackknife estimate = 21.57 ± 1.0; two-sample *t*-test: $p < .0001$). Several loci identified as part of the genetic basis for adaptation to osmotic stress were genes well known to be involved in responses to osmotic stress. These include *ompR* encoding a transcription factor critical for regulation of the osmotic stress response (Seo et al., 2017), *proP* encoding a H^+-symporter that senses osmotic shifts and responds by importing osmolytes (Racher et al., 1999), *treR* encoding a repressor of operons involved in trehalose metabolism under osmotic stress (Horlacher & Boos, 1997), and two genes (*rcsF* and *wcaJ*) involved in synthesis of the colanic acid capsule which is induced by osmotic shock (Sledjeski & Gottesman, 1996) and can provide a protective effect against osmotic stress (Chen et al., 2004). Similarly, loci identified as part of the genetic basis of adaptation to antibiotic stress contained several genes that are known to provide resistance to aminoglycosides including *fhuA* encoding the ribosomal elongation factor G, *fusA* encoding an outer membrane ferrichrome transporter, and genes involved in energy metabolism (intergenic region adjacent to *cyoA*, *nuoE*, *nuoG*, *nuoM*; Ibacache-Quiroga et al., 2018). Two loci were involved in adaptation to both antibiotic and osmotic stress: the uncharacterized protein *yeaR*, and *atpG* involved in the transport of H^+ in the ATP synthase complex. Supplementary Tables S3 and S4 provide the full lists with functional annotation of loci under selection for osmotic and antibiotic stress respectively.

Osmotic stress affects IS transposition rates

After experimental evolution, population-level genome sequencing showed that new IS insertions were present in all populations. IS10 was the most active IS with a total of 1136 new insertions, followed by IS1 (167 insertions), IS150 (52 insertions), IS186 (48 insertions), IS2 (43 insertions), IS5 (29 insertions), and IS4 (6 insertions). When exposed to osmotic stress, the transposition rate of four IS's was found to significantly differ from transposition rates in the control treatment (Figure 3). IS10 and IS186 had higher transposition rates under osmotic stress, while IS2 and IS150 had lower transposition rates under osmotic stress. IS10 transposition rates under osmotic stress were also significantly higher than the control treatment when not considering IS10 insertions in the

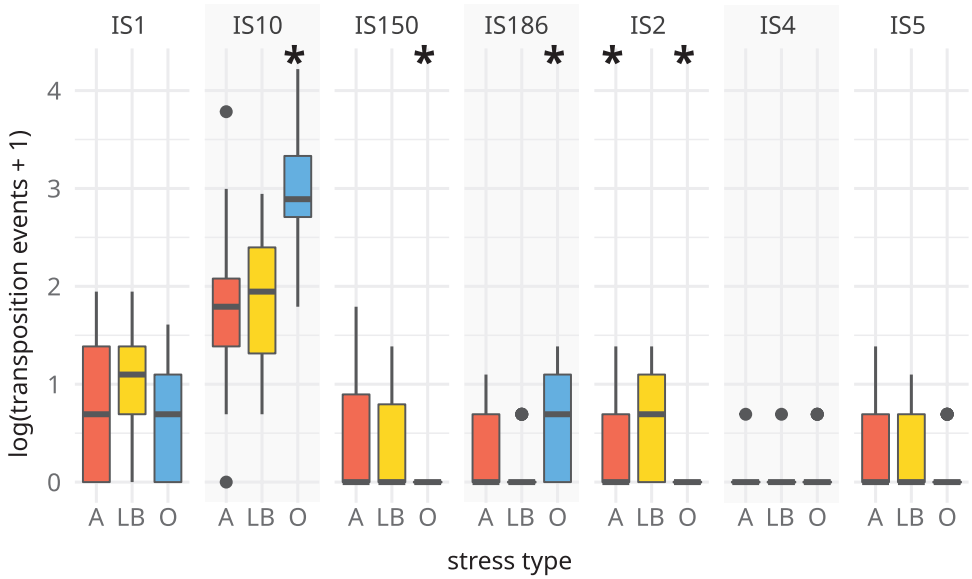

Figure 3. IS transposition rates. Log-transformed numbers of observed new IS insertions in E. coli populations after experimental evolution. Transposition counts are grouped by stress type (A = antibiotic stress, LB = control treatment, O = osmotic stress). An asterisk indicates that the number of transposition events for a given stress type is significantly different from the control treatment (Mann–Whitney *U*-test; $p < .05$). Black line in the boxplot indicates the median value, boxes span the 25th–75th percentile and vertical lines the maximum and minimum value. Dots indicate outliers.

mutS or *mutL* genes (two-sample *t*-test; $p < .001$). When exposed to antibiotic stress, IS2 had significantly lower transposition rates compared to the control treatment.

Discussion

This experiment investigated the rate of hypermutator emergence under different types and levels of stress. Our results provide clear evidence that the emergence of hypermutators is stress dependent, with hypermutator emergence increasing when exposed to osmotic stress but not when exposed to antibiotic stress. The observed increase in hypermutator emergence due to osmotic stress is in line with other studies where maladaptation due to an environmental stressor increased the rate of hypermutator emergence (Pal et al., 2007; Swings et al., 2017). The finding that, at least under our experimental conditions, the emergence of hypermutators is not affected by exposure to gentamicin furthermore nuances earlier reports on the evolution of increased mutation rates under antibiotic stress (Hammerstrom et al., 2015; Ibacache-Quiroga et al., 2018). Although in our experiment hypermutators frequently evolved when exposed to osmotic stress, hypermutator populations did not show a detectable increase in the rate or degree of adaptation to osmotic stress. This is in contrast with the abovementioned studies where hypermutators did seem to provide an advantage when adapting to a particular stress. Based on our results we identified three potential (non-exclusive) causes of stress dependent emergence of hypermutators: (a) difference in the size of the genetic basis for adaptation; (b) difference in stress levels; and (c) difference in the supply of hypermutators.

Analysis of the genetic basis for adaptation confirmed our presumption that more loci contribute to adaptation to osmotic stress compared to antibiotic stress. Furthermore, populations adapting to osmotic stress did not restore their growth parameter values to those observed in unexposed populations, while populations adapting to antibiotic stress did restore their growth parameter values. This situation, where adaptation to osmotic stress probably remained an ongoing process throughout the experiment while populations exposed to antibiotic stress were able to rapidly adapt, is expected to favor the emergence of hypermutators under osmotic stress but not under antibiotic stress (Giraud et al., 2001; Swings et al., 2017; Wielgoss et al., 2013).

Our results point towards a potential relation between the genetic architecture of adaptation to a particular stress and the probability of evolving hypermutability during adaptation. It should be noted that the size of the genetic basis for adaptation has been evaluated from loci mutated in parallel in sequence data obtained after 1,000 generations. This means that the genetic basis is likely to contain both mutations that directly contribute to adaptation to the stress, and mutations that compensate the negative pleiotropic effects of the first ones (Lenormand et al., 2018). This does not change the prediction of a positive link between the size of the genetic basis and the advantage to hypermutators. Indeed, Perron et al. (2010) have shown that hypermutators have an advantage for the compensatory part of the adaptation process. Incorporating the DFE into models of mutation rate evolution might be important to gain a better understanding. Based on our results, we hypothesize that hypermutators are more likely to emerge when adaptation occurs through many (potentially compensatory) small effect mutations compared to adaptation through a few large-effect beneficial mutations.

Although we tried to have equivalent stress levels for the two stress types used, osmotic stress often had a stronger effect on *E. coli* growth compared to antibiotic stress when this was measured in subsequent assays (Figure 2B). Therefore, stress type and stress levels might have been confounded in our experiment and we cannot exclude the possibility that the difference in emergence rate of hypermutators between the two stress types is at least partly due to a difference in stress levels. This could also explain the apparent disagreement between our results and those of Ibacache-Quiroga et al. (2018), who found that in *E. coli* populations exposed to gentamicin hypermutators often emerge and that hypermutators adapt faster, indicating a potential benefit of increased mutation rates when adapting to gentamicin.

However, their populations were experimentally evolved in a chemostat with continuously increasing concentrations of gentamicin (from 0.03 to 256 µg/mL), thus maintaining populations at a relatively constant level of maladaptation to their environment, which is expected to favor high mutation rates. Interestingly, they found a high degree of parallelism in adaptation (both for mutators and nonmutators) involving mutations in five common genetic elements appearing in the same order related to gentamicin concentration (respectively *fusA, cyoABCDE, potABCD, fhuA*, and *atpABCDEFGHI*). This indicates that the DFE might be dependent on gentamicin concentration with potentially a higher number of loci that contribute to adaptation at higher gentamicin concentrations. Indeed, in line with what is expected at low gentamicin concentrations, mostly *fusA* was mutated in our experimental populations exposed to antibiotic stress, while resistance mutations in the other genetic elements detected in Ibacache-Quiroga et al. (2018) only sporadically appeared. It is thus possible that there is a gentamicin concentration-dependent advantage of hypermutators influencing emergence, but with the range of concentrations in our experiment we were not able to detect this.

Based on our experimental observation of increased IS10 transposition rates when exposed to osmotic stress, we identified a third factor that potentially contributed to stress dependent emergence of hypermutators. An up-regulation of the activity of mobile genetic elements due to environmental stress has been shown in many organisms (Fitzgerald & Rosenberg, 2019) and particularly in bacteria (Vandecraen et al., 2017). IS10 transposition rate is known to increase with exposure to UV light (Eichenbaum & Livneh, 1998), but to the best of our knowledge this has not yet been documented when exposed to osmotic stress. Increase in mobile genetic element (MGE) transposition rate in stressful environments has been proposed to be advantageous because it facilitates and accelerates adaptation under adverse conditions (Fitzgerald & Rosenberg, 2019). The argument shares strong similarities with the advantage of hypermutators in stressful environments, except that (a) the mutation rate is directly environmentally regulated, instead of implying a second order selection on the genetic determinants of mutation rate and (b) the nature of mutations is different: hypermutators increase SNPs and 1bp indels whereas MGE transposition causes either disruptive mutations or changes in the expression level of the gene upstream of which the MGE inserts. It is important to note that our results (Figure 3) as well as other studies (e.g., Maharjan and Ferenci, 2017) indicate that different IS families have different sensitivities of their transposition rate to different stresses and that there are cases of reduction of transposition rate in stressful environments. The evolutionary forces and mechanisms acting on the environmental sensitivity of transposition rate are likely more complex than those acting on mutation rate evolution because MGE's and the genomes carrying them often have different evolutionary interests.

Adaptive benefits of IS10 insertions under osmotic stress have been described in sigma factor RpoS deficient *E. coli*, where an IS10 insertion in the promoter of the RpoS-dependent *otsBA* operon rewires expression, making it RpoS-independent and partially restores its functionality for osmoregulation (Stoebel et al., 2009). However, IS10-free RpoS deficient strains were also able to adapt to osmotic stress but not through restoring *otsBA* expression. The authors conclude that increased mutation rates at certain loci due to IS insertions, rather than unique phenotypic effects caused by IS insertions, cause IS-mediated mutations to fix in populations (Stoebel & Dorman, 2010). Along the same line, populations in the control and antibiotic stress treatments in our experiment predominantly became hypermutator through other mutational routes than IS10 insertions in *mutS* and *mutL*, which probably reflects differences in IS10-mediated mutation rates. It is however difficult to estimate the quantitative effect of increased hypermutator-inducing mutation rates through IS10 transposition on the emergence of hypermutators. There is thus a possibility that increased IS10 transposition rates contributed to a higher emergence of hypermutators in populations exposed to osmotic stress. There are indications that a similar mechanism played a role in hypermutator emergence in experimental *Vibrio splendidus* populations exposed to osmotic stress, where the excision of a mobile genetic element present in *mutS* induced a hypermutator phenotype (Chu et al., 2017).

Chao and McBroom (1985) indicate that IS10 itself acts as a mutator gene in *E. coli*, as its advantage in competition with IS10-free strains is frequency-dependent and linked to new IS10 transpositions. Furthermore, Consuegra et al. (2021) observed an overall lower number of IS-mediated mutations in hypermutator populations of the LTEE experiment. They hypothesize that there is an indirect benefit associated with hypermutability by preventing the increase and fixation of IS-mediated mutations. These studies, in addition to our results, point towards multiple complex interactions between IS-mediated mutations, evolvability, and the evolution of mutation rate.

Overall, the results of this study contribute to a better understanding of the evolution of mutation rates in bacteria during adaptation by showing that the emergence of hypermutators differs depending on the type of stress. Our experiment highlighted multiple factors potentially associated with specific stress types (size of genetic basis for adaptation, stress levels, and supply of hypermutators) contributing to the observed differences. Future experiments are needed to estimate the relative contribution of each factor to hypermutator emergence. It is critical to gain an in-depth understanding of drivers for hypermutability, as increased mutation rates are often involved in public health issues such as virulence acquisition, chronic infections, foodborne disease, and antibiotic resistance (Jolivet-Gougeon et al., 2011).

Supplementary material

Supplementary material is available online at *Evolution Letters* (https://academic.oup.com/evlett/qrad019).

Data availability

Raw sequencing data (fastq files) are deposited on ENA (project PRJEB60622). Data on mutator emergence, population parameters (growth kinetics and OD values) and the list of all the mutations detected in the experimentally evolved populations are on Dryad (doi:10.5061/dryad.hqbzkh1mr).

Author contributions

M.C., C.J.R., and S.B. conceived the project. M.C., C.J.R., M.F., F.G., L.P., M.-P.D., and S.B. performed the experimental evolution. M.F., F.G., L.S., and J.H. characterized the evolved populations. M.C. and S.B. analyzed the data. M.C. and S.B. wrote the manuscript.

Conflict of interest: The authors declare no conflict of interest.

Acknowledgments

This work was supported by the European Research Council HGTCODONUSE (ERC-2015-CoG-682819) to S.B. We thank two anonymous reviewers for their constructive comments.

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