Current Biology 22, 1235–1240, July 10, 2012 ©2012 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2012.04.056

Report

The Evolution of Low Mutation Rates in Experimental Mutator Populations of *Saccharomyces cerevisiae*

Michael J. McDonald,[1,*] Yu-Ying Hsieh,[1,2] Yen-Hsin Yu,[1] Shang-Lin Chang,[1,3] and Jun-Yi Leu[1]
[1]Institute of Molecular Biology, Academia Sinica, Taipei 115, Taiwan
[2]Department of Life Sciences and Institute of Genome Sciences, National Yang-Ming University, Taipei 115, Taiwan
[3]Molecular Cell Biology, Taiwan International Graduate Program, Institute of Molecular Biology, Academia Sinica, and Graduate Institute of Life Sciences, National Defense Medical Center, Taipei 115, Taiwan

Summary

Mutation is the source of both beneficial adaptive variation and deleterious genetic load, fueling the opposing selective forces than shape mutation rate evolution. This dichotomy is well illustrated by the evolution of the mutator phenotype, a genome-wide 10- to 100-fold increase in mutation rate. This phenotype has often been observed in clonally expanding populations exposed to novel or frequently changing conditions [1–5]. Although studies of both experimental and natural populations have shed light on the evolutionary forces that lead to the spread of the mutator allele through a population [5–11], significant gaps in our understanding of mutator evolution remain [12]. Here we use an experimental evolution approach to investigate the conditions required for the evolution of a reduction in mutation rate and the mechanisms by which populations tolerate the accumulation of deleterious mutations. We find that after ~6,700 generations, four out of eight experimental mutator lines had evolved a decreased mutation rate. We provide evidence that the accumulation of deleterious mutations leads to selection for reduced mutation rate clones in populations of mutators. Finally, we test the long-term consequences of the mutator phenotype, finding that mutator lines follow different evolutionary trajectories, some of which lead to drug resistance.

Results and Discussion

While the invasion of mutator alleles has been observed upon multiple occasions [5, 11] and antimutator alleles have been identified [13, 14], the restoration of low mutation rates in populations fixed for the mutator phenotype has yet to be demonstrated in experimental populations—even those that have been propagated for 50,000 generations [15] (although see [16, 17]). One of the most consistent results of experimental evolution is the steep initial increase in the fitness of a population, a response to the typically novel conditions inflicted upon the focal organism by experimenters [15, 18]. Under these conditions, low mutation rates and low fitness increase the likelihood of fixation of a mutator allele by hitchhiking with a new beneficial mutation [12, 19]. Here we test the expectation that the opposite circumstances—an initially high mutation rate in a high-fitness population—could induce selective pressure against high mutation rates. To create these conditions, we engineered a *msh2* haploid mutator strain of *Saccharomyces cerevisiae* and propagated replicate populations in an environment to which they were already relatively well adapted.

Fitness Trajectories Suggest Weak Selection

In order to determine the fitness trajectory of the eight mutator and eight nonmutator lines during experimental evolution, we obtained the measurements for fitness relative to the corresponding ancestral strain of populations at generations 500, 1,800, 3,100, 4,700, 5,700, and 6,700 (Figure 1). The shallow increase in fitness shown in Figure 1 contrasts with the initially steep, then decelerating increase in fitness observed in other evolution experiments [15, 18] and support the idea that the founding populations were initially relatively well adapted. The nonmutator lines show a steady increase in fitness over the course of the experiment with four of the eight lines showing, after 6,700 generations, a significant increase in absolute growth rate compared to the ancestor (Figure 1, 95% confidence interval, n = 4). The difference in the fitness trajectory of our experimental populations compared to other experiments suggests that these populations were experiencing weak directional selection. Five out of eight mutator lines (compared to one of the nonmutator lines) had a lower growth rate than the ancestor at some point during the experiment (Figure 1, 95% confidence interval, n = 4). Fitness decreases, or even population extinction, may occur in mutation accumulation experiments [20]; that the absolute growth rates of some mutator populations decreased at some point during the course of the experiment suggests that the mutator phenotype had a deleterious effect upon populations.

Four Experimental Mutator Lines Evolved a Decreased Mutation Rate

In order to determine whether the deleterious effect of the mutator phenotype was associated with the evolution of decreased mutation rates, we performed fluctuation tests [21] on the mutator and nonmutator experimental lines (see Supplemental Experimental Procedures, available online, for details). Four out of eight mutator populations had a significant reduction in their mutation rate (as determined by nonoverlapping 95% confidence intervals). While there was also a general trend toward a decrease in the mutation rate of the nonmutator experimental lines, the reduced power of the assay for distinguishing between lower mutation rates meant it was difficult to discern any significant difference (Figures 2A and 2B).

The Decrease in Fitness of Mutator Lines Is Followed by a Decrease in the Mutation Rate

The forces behind the invasion of a mutator allele in asexual populations are well understood. Although a newly arising mutator allele may not confer any beneficial effect per se, such an allele can increase in frequency because it has a higher probability than a nonmutator of being linked with a beneficial mutation [22, 23]. However, for a newly arising reduced mutation rate allele, not only is it unlikely to confer any benefit itself,

*Correspondence: mikejmcdonald@yahoo.com

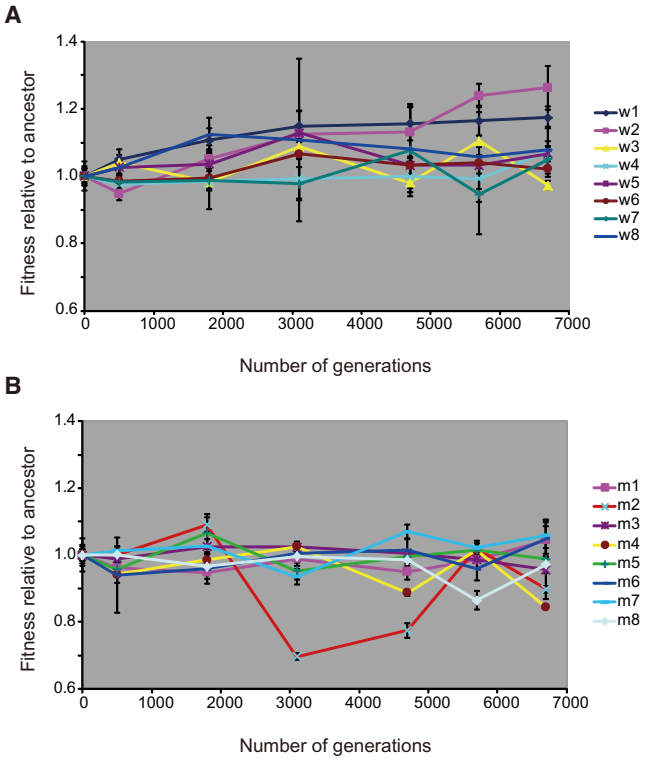

Figure 1. Fitness Trajectories of the Nonmutator and Mutator Lines during 6,700 Generations of Experimental Evolution

(A) Nonmutator lines. (B) Mutator lines. Fitness is taken as the maximum growth rate achieved during 24 hr of growth (see Experimental Procedures). Four replicate cultures were used per strain. Data represent the mean ± 95% confidence intervals. See also Figures S2 and S3.

but it is also less likely than the mutator-majority to be associated with a new beneficial mutation. Instead, its advantage may derive from the decreased probability of association with deleterious mutations. Indirect selection for nonmutators is predicted to lead to no net change in the population fitness trajectory. This is because as the mutator clones accumulate deleterious mutations, they should decrease in fitness and drop from the population while the least mutation-loaded clones increase in frequency, without a net change in population fitness. A direct selection model would posit the opposite—that an increase in fitness should be associated with the emergence of the reduced mutation rate clone. The final, and least likely, possibility is that a reduced mutator allele hitchhikes with a beneficial mutation.

We measured a mutation rate trajectory for two lines (m2 and m8), carrying out fluctuation tests using independent clones obtained at regular time intervals during the evolution experiment. A decrease in the mutation rate occurred between generations 3,100 and 4,700 in line m2, and between generations 5,700 and 6,700 in line m8, both with a significant decrease in fitness in the interval immediately preceding it (Figures 1, 2C, and 2D). Interestingly, the two clones used for the assessment of line m2 at generation 3,100 were found to have different mutation rates (Figure S1).

Fitness Dynamics of Emergence of Reduced Mutation Rates in Mutator Populations

Theory predicts that a population experiencing weak selection and high mutation rate should accumulate population-wide variation. Conversely, a fixation event should be accompanied by a reduction in genetic variation throughout the population. To test these predictions we sampled 96 individuals from each of five time points for lines m2 and m8 (Figure S2), using the within-population fitness variance as a proxy for genetic variation. We found that while fitness variance tended to increase over time, populations sampled at time points in agreements with the fixation of reduced mutation rate alleles in both lines showed bottlenecks, supporting the notion of recent selective sweeps.

It is possible that the spread of the reduced mutation rate was due to direct selection rather than its reduced likelihood of sustaining deleterious mutations. In order to directly test the ability of the reduced mutation rate clones to invade mutator populations, we carried out competitive fitness assays between differently marked individuals taken from different time points in lines m8 and m2. In line m8 we found that the reduced mutation rate clone taken from generation 6,700 had a 1.19-fold fitness (±0.04, 95% confidence interval) advantage compared to the mutator clone taken from the same line at generation 5,700 (Figure 3). The discovery of both mutator and reduced mutation rate clones in the m2 population at generation 3,100 (Figure S1) afforded the opportunity to test for the ability of the reduced mutation rate clone to invade a contemporaneous mutator clone. When these two clones were competed the reduced mutation rate clone was found to have a 1.46-fold (±0.1, 95% confidence interval) fitness advantage over the mutator clone (Figure 3). It should be noted that 95% confidence intervals are a conservative estimate of the difference between two means and overlapping intervals do not automatically mean that two groups are not significantly different. These results suggest that it was not indirect selection that drove the reduced mutation rate clones to fixation in these two lines. Rather, the discovery of such a large difference in fitness between contemporaneous mutator and reduced mutation rate clones suggests that a direct fitness effect of the reduced mutator allele may have been responsible. However, the fact that population m2 appears to have had the larger proportion of its fitness increased after it had become fixed for the reduced mutation rate allele is evidence in support of the hitchhiking of the reduced mutation rate allele with another beneficial mutation. It is certainly impossible to rule out either of direct selection or hitchhiking, or indeed that they are mutually exclusive. The possibility that, in a population saddled with genetic load, a reduced mutation rate genotype could be more likely to be fixed by hitchhiking with a new beneficial mutation than a mutator genotype could prove worth investigating.

Experimental Lines Became Diploid in All Mutator Strains and Half of Wild-type Strains

Experimental propagation of haploid yeast cells has been shown to lead to a switch to the diploid state in as few as 600 generations [24]. We checked the DNA content of all mutator and nonmutator lines and found that while all mutator lines had become diploid sometime between 3,100 and 4,700 generations, after 6,700 generations, half of the nonmutator lines remained in the haploid state (Table S1). Diploids arise by endomitosis—the replication of the genome without a subsequent cell division. Such a genome doubling is unlikely to provide much respite from the mutations that had been accumulated so far. All deleterious mutations will be duplicated along with the rest of the genome and will still have a deleterious effect on the diploid. The advantage of

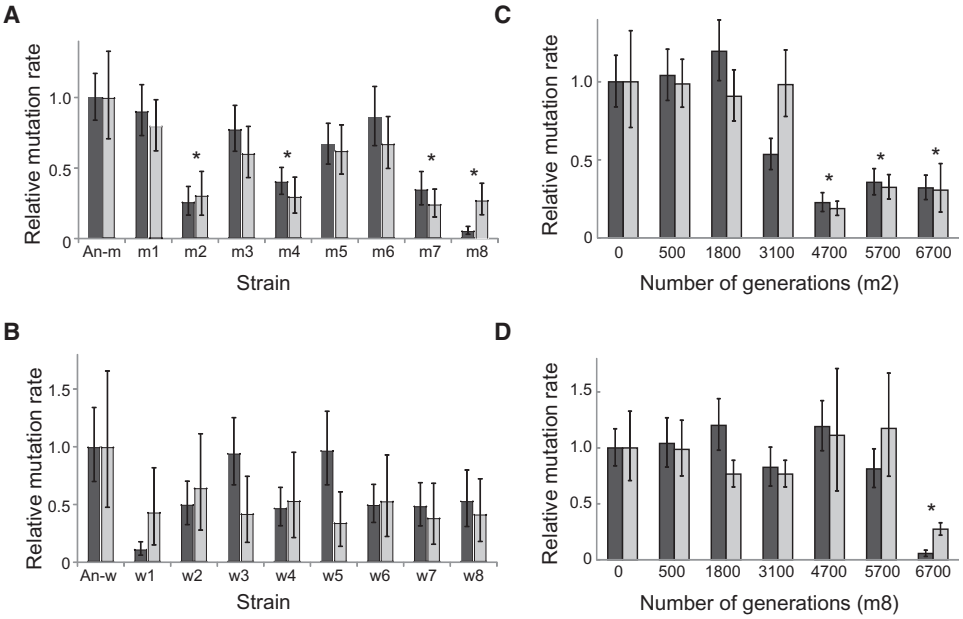

Figure 2. Mutation Rates of Ancestral and Evolved Strains

Two clones were chosen from each population, transformed with either the adenine (light) or the Clonat (dark) marked plasmids, and then fluctuation tests were performed using at least 16 cultures. Relative mutation rates of mutator (A) and nonmutator (B) strains collected from generation 6,700 are shown. (C and D) The mutation rate trajectory during the course of experimental evolution for lines m2 and m8, respectively. Asterisks indicate experimental lines with mutation rates significantly lower than the progenitor strain. Data represent the mean ± 95% confidence intervals. See also Figure S1.

diploidization for a mutator is also probably derived from the prevention of the deleterious effects of future mutations—not those that have already accumulated. The difference between the mutator and nonmutator experimental lines suggests stronger selective pressure for diploidy in the mutator lines. Interestingly, despite this signature of selection, there was never any increase in fitness following a ploidy change in the nonmutator lines and no association between diploidization and fitness increases in the mutator lines (fishers exact test, $p = 0.11$). Although not conclusive, these results are consistent with indirect, rather than direct, selection for ploidy change. Another possibility is that nontransitive fitness differences across populations combined with direct selection could produce the same fitness dynamics. However, the competition assays performed above (see also Figure S3) indicate that the direction and magnitude of between-population fitness differences were similar to those measured using growth assays.

The Mechanistic Basis of the Reduced Mutation Rate Phenotype

We next sought to investigate the molecular mechanisms by which the low mutation rate was restored. When a functional *MSH2* allele was reintroduced into mutator strains that had experienced a decrease in mutation rate (m2, m4, m7, and m8), the mutation rate was decreased to a value not significantly different from that of the wild-type for m4 and m8 (95% confidence interval, Figure S4). This result suggests that in lines m2 and m7, the mismatch repair pathway may have been affected by epistatic genetic interactions, rendering wild-type Msh2 incompatible.

Performing classical genetic analyses using our evolved lines was difficult as none of the derived strains were able to undergo sporulation, probably due to their carrying a large number of recessive deleterious mutations. As an alternative approach, we compared the expression profile of an evolved m8 clone (derived from generation 6,700) with its mutator ancestor, and as a control, an evolved w1 clone (derived from generation 6,700) to its nonmutator ancestor. The sets of underexpressed (<0.5-fold) and overexpressed (>2-fold) genes were quite distinct for the experimental and control assays with only a single gene overlap in the sets of overexpressed genes (414 genes, evolved m8), and no overlap in the sets of underexpressed genes (23 genes, evolved m8). In contrast, a repeat of the m8/mutator ancestor experiment saw a 94% overlap for over- and underexpressed genes. We compared this set of over- and underexpressed genes with transcription sets obtained from DNA damaging conditions such as MMS [25] and ionizing radiation [26], but found almost no overlap between these conditions. Five DNA replication initiation factors were overexpressed; two of these (Pol1 and Pol12) are essential components of the alpha/primase replication complex, the other two (Orc1 and Cdc45) are required for the binding of the replication complex to replication initiation sites, and Clb6 promotes early initiation of DNA synthesis [27]. It is tempting to speculate that the overexpression of replication initiation genes together with the highly processive DNA polymerase δ (encoded by Pol3) and the PCNA replication clamp could promote earlier and stronger initiation at replication origins in the evolved m8 line. Indeed, when human Orc1 alone is overexpressed in fission yeast, DNA replication becomes constitutive [28]. It has been demonstrated, both in yeast and in other species, that the mutation rate is lowest close to replication origins, especially those that fire earlier in S phase [29–31]. Oxidative lesions, if left unrepaired by MMR, can result in the DNA replication fork stalling. The gaps left by this process can either be repaired by high-fidelity homologous recombination (early in S phase) or low-fidelity translesion polymerases (late S phase) [30–32]. It may be

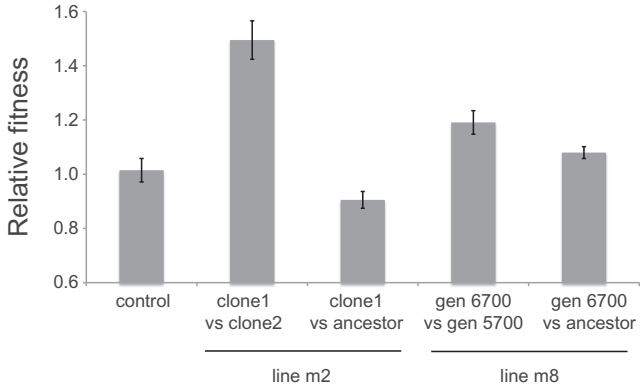

Figure 3. Competitive Fitness Assays between Mutators and Reduced Mutation Rates

From line m2, clone 1 (reduced mutation rate) is fitter than clone 2 (a mutator) from the m2 population at 3,100 generations (see also Figure S1). However, the reduced mutation rate clone 1 has a lower fitness than the founding mutator strain. From line m8, a clone taken at generation 5,700 (mutator) is less fit than a reduced mutation rate clone taken from generation 6,700. As a control, ancestral and derived clones were transformed with a plasmid identical except for (intact) *ADE2* or Clonat marker genes. When clones with different marker plasmids were competed, no difference in relative fitness was detectable. The control shown is two differently marked mutator ancestor clones; data shown represent the mean ± 95% confidence intervals.

that a reduction in mutation rate could be achieved by the more frequent use of homologous recombination rather than error-prone polymerase to fill lesions that persist into S phase due to a lack of mismatch repair.

Evolved Mutator Strains Have Varying Dependence on the Heat Shock Protein Hsp90

A predicted consequence of accumulated deleterious mutations is the evolution of enhanced physiological buffering for unstable proteins [20]. The most well-known buffering system is the heat shock protein Hsp90, its yeast homolog encoded by two genes, *HSC82* and *HSP82*. Contrary to expectations, the expression microarray analysis of the m8 line did not detect the overexpression of any yeast chaperone protein. In order to investigate the importance of Hsp90 to the evolved lines, we tested the effects of the Hsp90 inhibitor Macbecin II. This drug is expected to be harmful to cells that have sustained a large number of mutations because it will remove the buffering effect of Hsp90, unmasking the effects of deleterious mutations. Figure 4A shows that, in general, nonmutator experimental lines grew better in the presence of Macbecin II relative to their progenitor, while mutator lines showed a variable response to the drug.

The nonmutator evolved lines showed a positive correlation between growth in YPD and growth in YPD + Macbecin II (Figure 4B), indicating that improved rates of growth are not Macbecin II specific. Conversely, the mutator strains show no such correlation (Figure 4C). The finding that mutator populations in this study exhibit varying dependence on heat shock protein buffering systems suggests that alternative physiological buffering mechanisms or compensatory mutations may be able to ameliorate the effects of mutation accumulation. It is of note that the only two lines to have a significant decrease in fitness (Figure 4, 95% confidence interval) when treated with Macbecin II were lines m3 and m6, two lines that did not evolve a decrease in mutation rate. This suggests

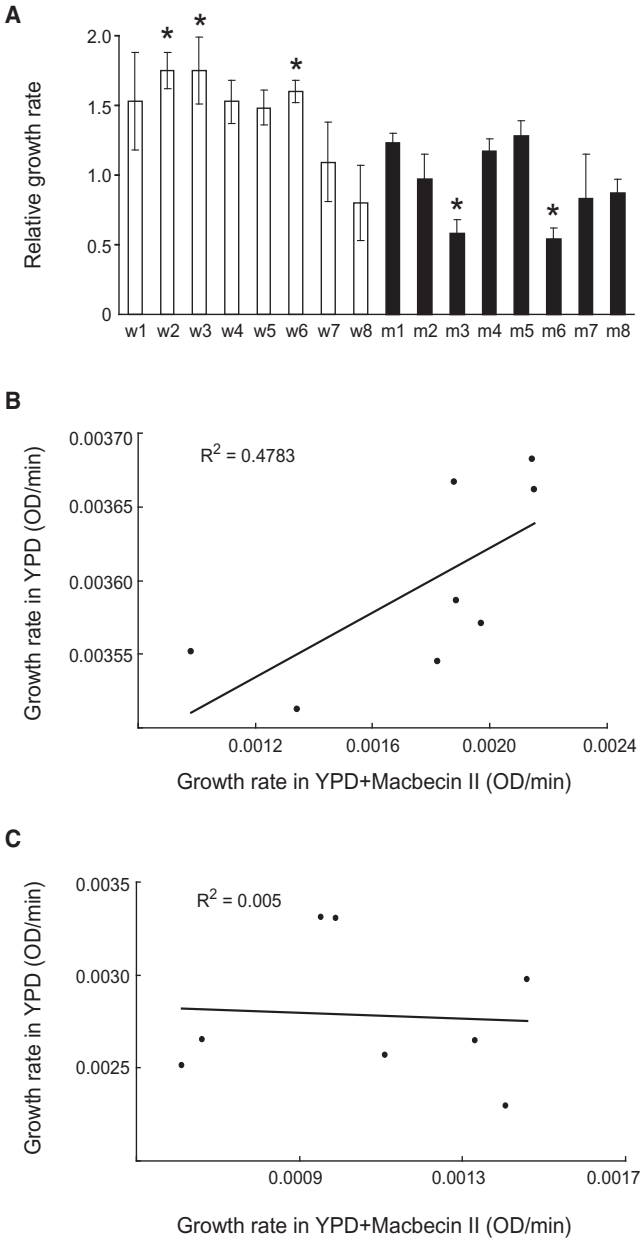

Figure 4. Growth of Experimental Lines in the Presence of the Hsp90-Inhibiting Drug Macbecin II

(A) The growth of evolved strains relative to their progenitor strain in the presence of Macbecin II. Asterisks indicate significant difference to the ancestor (95% confidence interval).

(B and C) The growth of nonmutator lines (C) in the presence of Macbecin II was correlated with performance in YPD media without Macbecin II (Pearson's correlation coefficient $r = +0.69$, $p = 0.028$, one-tailed), while (C) growth of the mutator strains in YPD + Macbecin II showed no correlation with growth in YPD (Pearson's correlation coefficient, $r = -0.07$, $p = 0.43$).

that these lines may tolerate the accumulation of deleterious mutations by depending on the buffering effects of heat shock protein.

The mutator phenotype has long been hypothesized to play an important role in cancer progression [1]. Mutation shock therapies, implemented using drugs such as Macbecin II, have been employed to unmask the deleterious mutations accumulated by cancer cells. Our results indicate that even

cancerous growths that have accumulated many mutations may have varying sensitivities to such drugs.

Conclusions

In this study we have observed the restoration of low mutation rates during the extended propagation of mutator strains in an environment to which they are well adapted. Remarkably, this occurred without the advantage that natural populations have of gene flow from outside the population to restore the *msh2* mutant via recombination. These results hint that interactions between mutation rates and genetic load may have unexpected effects upon mutation rate dynamics and that cells have a variety of mechanisms by which they adapt to the accumulation of deleterious mutations.

Experimental Procedures

Long-Term Experimental Evolution of Yeast Populations

The parental *S. cerevisiae* strain is isogenic with *W303* (*MATa his3-11,15 leu2-3,112 trp1-1 ade2-1 hmlΔ::TRP1*). The *HML* locus was deleted to eliminate the possibility of mating type switching during the evolution experiment. The mutator strain was created by replacing the *MSH2* gene from the haploid strain with either a G418- or a hygromycin-resistance gene. The wild-type strain was modified by insertion of either a G418- or a hygromycin-resistance gene in the *HO* locus. Eight experimental lines were established for the wild-type strain and the *msh2* deletion mutator strain. Lines 1–8 for both strains were alternatively marked with one of the two drug-resistant markers, each drug marker containing one of four different genetic bar codes. These steps were taken to ensure there was no cross-contamination during passaging of the lines, and all lines were confirmed by PCR as having no cross-contamination after 6,700 generations. Each cycle comprised a daily 10,000-fold dilution in 3 ml of YPD in test tubes (a bottleneck of 3×10^4 cells). Once every ten transfers, population samples from each line were stored in 20% glycerol at −80°C.

Supplemental Information

Supplemental Information includes four figures, one table, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2012.04.056.

Acknowledgments

We thank members of the Leu lab for helpful discussions and G. Lang, H.J.E. Beaumont, T.F. Cooper, and three anonymous reviewers for comments on the manuscript. We thank the Microarray Core of IMB for microarray support. M.J.M. was funded by an Academia Sinica distinguished postdoctoral fellowship. J.Y.L. was supported by Academia Sinica of Taiwan (grant no. AS23-17) and National Science Council of Taiwan (grant no. NSC99-2321-B-001-031).

Received: October 14, 2011
Revised: February 1, 2012
Accepted: April 26, 2012
Published online: June 21, 2012

References

1. Bielas, J.H., Loeb, K.R., Rubin, B.P., True, L.D., and Loeb, L.A. (2006). Human cancers express a mutator phenotype. Proc. Natl. Acad. Sci. USA *103*, 18238–18242.
2. Denamur, E., Lecointre, G., Darlu, P., Tenaillon, O., Acquaviva, C., Sayada, C., Sunjevaric, I., Rothstein, R., Elion, J., Taddei, F., et al. (2000). Evolutionary implications of the frequent horizontal transfer of mismatch repair genes. Cell *103*, 711–721.
3. Giraud, A., Matic, I., Tenaillon, O., Clara, A., Radman, M., Fons, M., and Taddei, F. (2001). Costs and benefits of high mutation rates: adaptive evolution of bacteria in the mouse gut. Science *291*, 2606–2608.
4. LeClerc, J.E., Payne, W.L., Kupchella, E., and Cebula, T.A. (1998). Detection of mutator subpopulations in Salmonella typhimurium LT2 by reversion of his alleles. Mutat. Res. *400*, 89–97.
5. Pal, C., Maciá, M.D., Oliver, A., Schachar, I., and Buckling, A. (2007). Coevolution with viruses drives the evolution of bacterial mutation rates. Nature *450*, 1079–1081.
6. Funchain, P., Yeung, A., Stewart, J.L., Lin, R., Slupska, M.M., and Miller, J.H. (2000). The consequences of growth of a mutator strain of Escherichia coli as measured by loss of function among multiple gene targets and loss of fitness. Genetics *154*, 959–970.
7. Miller, J.H., Suthar, A., Tai, J., Yeung, A., Truong, C., and Stewart, J.L. (1999). Direct selection for mutators in Escherichia coli. J. Bacteriol. *181*, 1576–1584.
8. Montanari, S., Oliver, A., Salerno, P., Mena, A., Bertoni, G., Tümmler, B., Cariani, L., Conese, M., Döring, G., and Bragonzi, A. (2007). Biological cost of hypermutation in Pseudomonas aeruginosa strains from patients with cystic fibrosis. Microbiology *153*, 1445–1454.
9. Morgan, A.D., Bonsall, M.B., and Buckling, A. (2010). Impact of bacterial mutation rate on coevolutionary dynamics between bacteria and phages. Evolution *64*, 2980–2987.
10. Oliver, A., and Mena, A. (2010). Bacterial hypermutation in cystic fibrosis, not only for antibiotic resistance. Clin. Microbiol. Infect. *16*, 798–808.
11. Sniegowski, P.D., Gerrish, P.J., and Lenski, R.E. (1997). Evolution of high mutation rates in experimental populations of E. coli. Nature *387*, 703–705.
12. Sniegowski, P.D., Gerrish, P.J., Johnson, T., and Shaver, A. (2000). The evolution of mutation rates: separating causes from consequences. Bioessays *22*, 1057–1066.
13. Fijalkowska, I.J., and Schaaper, R.M. (1995). Effects of Escherichia coli dnaE antimutator alleles in a proofreading-deficient mutD5 strain. J. Bacteriol. *177*, 5979–5986.
14. Schaaper, R.M., and Cornacchio, R. (1992). An Escherichia coli dnaE mutation with suppressor activity toward mutator mutD5. J. Bacteriol. *174*, 1974–1982.
15. Barrick, J.E., Yu, D.S., Yoon, S.H., Jeong, H., Oh, T.K., Schneider, D., Lenski, R.E., and Kim, J.F. (2009). Genome evolution and adaptation in a long-term experiment with Escherichia coli. Nature *461*, 1243–1247.
16. Tröbner, W., and Piechocki, R. (1984). Selection against hypermutability in Escherichia coli during long term evolution. Mol. Gen. Genet. *198*, 177–178.
17. Notley-McRobb, L., Seeto, S., and Ferenci, T. (2002). Enrichment and elimination of mutY mutators in Escherichia coli populations. Genetics *162*, 1055–1062.
18. Schoustra, S.E., Bataillon, T., Gifford, D.R., and Kassen, R. (2009). The properties of adaptive walks in evolving populations of fungus. PLoS Biol. *7*, e1000250.
19. Gerrish, P.J., Colato, A., Perelson, A.S., and Sniegowski, P.D. (2007). Complete genetic linkage can subvert natural selection. Proc. Natl. Acad. Sci. USA *104*, 6266–6271.
20. Maisnier-Patin, S., Roth, J.R., Fredriksson, A., Nyström, T., Berg, O.G., and Andersson, D.I. (2005). Genomic buffering mitigates the effects of deleterious mutations in bacteria. Nat. Genet. *37*, 1376–1379.
21. Luria, S.E., and Delbrück, M. (1943). Mutations of Bacteria from Virus Sensitivity to Virus Resistance. Genetics *28*, 491–511.
22. Smith, J.M., and Haigh, J. (1974). The hitch-hiking effect of a favourable gene. Genet. Res. *23*, 23–35.
23. Barton, N.H. (2000). Genetic hitchhiking. Philos. Trans. R. Soc. Lond. B Biol. Sci. *355*, 1553–1562.
24. Gerstein, A.C., Chun, H.J.E., Grant, A., and Otto, S.P. (2006). Genomic convergence toward diploidy in Saccharomyces cerevisiae. PLoS Genet. *2*, e145.
25. Jelinsky, S.A., and Samson, L.D. (1999). Global response of Saccharomyces cerevisiae to an alkylating agent. Proc. Natl. Acad. Sci. USA *96*, 1486–1491.
26. Gasch, A.P., Huang, M.X., Metzner, S., Botstein, D., Elledge, S.J., and Brown, P.O. (2001). Genomic expression responses to DNA-damaging agents and the regulatory role of the yeast ATR homolog Mec1p. Mol. Biol. Cell *12*, 2987–3003.
27. Caldwell, J.M., Chen, Y.H., Schollaert, K.L., Theis, J.F., Babcock, G.F., Newlon, C.S., and Sanchez, Y. (2008). Orchestration of the S-phase and DNA damage checkpoint pathways by replication forks from early origins. J. Cell Biol. *180*, 1073–1086.
28. Wolf, D.A., Wu, D., and McKeon, F. (1996). Disruption of re-replication control by overexpression of human ORC1 in fission yeast. J. Biol. Chem. *271*, 32503–32506.

29. Lang, G.I., and Murray, A.W. (2011). Mutation rates across budding yeast chromosome VI are correlated with replication timing. Genome Biol. Evol. *3*, 799–811.
30. Chuang, J.H., and Li, H. (2004). Functional bias and spatial organization of genes in mutational hot and cold regions in the human genome. PLoS Biol. *2*, E29.
31. Stamatoyannopoulos, J.A., Adzhubei, I., Thurman, R.E., Kryukov, G.V., Mirkin, S.M., and Sunyaev, S.R. (2009). Human mutation rate associated with DNA replication timing. Nat. Genet. *41*, 393–395.
32. Waters, L.S., and Walker, G.C. (2006). The critical mutagenic translesion DNA polymerase Rev1 is highly expressed during G(2)/M phase rather than S phase. Proc. Natl. Acad. Sci. USA *103*, 8971–8976.