4-12-2017

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Recommended Citation
Skelly, Daniel A.; Magwene, Paul M.; Meeks, Brianna; and Murphy, Helen A., Known mutator alleles do not markedly increase mutation rate in clinical Saccharomyces cerevisiae strains (2017). PROCEEDINGS OF THE ROYAL SOCIETY B-BIOLOGICAL SCIENCES, 284(1852). 10.1098/rspb.2016.2672
Known mutator alleles do not markedly increase mutation rate in clinical *Saccharomyces cerevisiae* strains

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Natural selection has the potential to act on all phenotypes, including genomic mutation rate. Classic evolutionary theory predicts that in asexual populations, mutator alleles, which cause high mutation rates, can fix due to linkage with beneficial mutations. This phenomenon has been demonstrated experimentally and may explain the frequency of mutators found in bacterial pathogens. By contrast, in sexual populations, recombination decouples mutator alleles from beneficial mutations, preventing mutator fixation. In the facultatively sexual yeast *Saccharomyces cerevisiae*, segregating alleles of *MLH1* and *PMS1* have been shown to be incompatible, causing a high mutation rate when combined. These alleles had never been found together naturally, but were recently discovered in a cluster of clinical isolates. Here we report that the incompatible mutator allele combination only marginally elevates mutation rate in these clinical strains. Genomic and phylogenetic analyses provide no evidence of a historically elevated mutation rate. We conclude that the effect of the mutator alleles is dampened by background genetic modifiers. Thus, the relationship between mutation rate and microbial pathogenicity may be more complex than once thought. Our findings provide rare observational evidence that supports evolutionary theory suggesting that sexual organisms are unlikely to harbour alleles that increase their genomic mutation rate.

1. Introduction

Mutation rates, like most organismal phenotypes, are subject to natural selection [1]. Alleles that increase the genomic mutation rate can not only be subject to direct selection but also to indirect selection on the fitness effects of mutations at other loci. A rich body of evolutionary theory predicts that in asexual populations, mutator alleles, which cause high mutation rates, can fix due to linkage with beneficial mutations. The probability of mutator hitchhiking is related to the supply and magnitude of such mutations, as well as the population size [2–6]. Experimental studies with *Escherichia coli* [7–12] and *Saccharomyces cerevisiae* [13–15] have provided evidence for this phenomenon, and clinical isolates of asexual pathogenic microbes have been shown to contain elevated frequencies of mutator strains [16–20], suggesting that mutator alleles have a propensity to rise in frequency during invasion of a new ecological niche. In contrast with asexual populations, mutator hitchhiking is not predicted to occur in sexual organisms. According to simulations and theoretical models, sex and recombination will erode linkage, separating beneficial mutations from the mutator alleles that caused them and preventing an increase in frequency of the mutators [5,21–25]. This prediction has garnered experimental support in *S. cerevisiae* [26]. However, some population genetic models have shown that under certain restrictive circumstances, fluctuating environments could select for mutator alleles in sexual populations [27–29].
Sexual microbes in the real world are mostly eukaryotes with facultatively sexual life cycles that undergo infrequent sex between periods of clonal growth [30]. The degree of linkage within the genome, as well as the strength and direction of selection and environmental change, are usually unknown. Thus, it is unclear whether natural populations of sexual microbes are expected to contain considerable frequencies of mutator strains, and to our knowledge none have been reported.

The budding yeast *S. cerevisiae* has been collected from locations and ecological settings around the world [31–34]. Its life cycle can include asexual growth in haploid and diploid forms, as well as both outcrossing and extreme inbreeding (matting-type switching and intra-ascus mating) [35]. Outcrossing rates are inferred to be extremely low in natural populations, with most growth occurring clonally in the diploid phase [36,37]. *Saccharomyces cerevisiae* is also known to be an opportunistic pathogen [38,39]. Pathogenic isolates have been shown to have high levels of heterozygosity [40], suggesting that sexual outcrossing events prior to colonization of a new ecological niche may be associated with opportunistic pathogenicity. The global population of *S. cerevisiae* contains two alleles in the genes MLH1 (G761D) and PMS1 (R818 K) [41] that, when engineered together in a laboratory strain background, increase the mutation rate 20–400× above wild-type [42]. Mlh1p and Pms1p form a heterodimer that plays a major role in the process of DNA mismatch repair, a system for identifying and correcting mutations that occur during replication and recombination [43]. The mutation rate-increasing substitutions are found within the domains that mediate the Mlh1p–Pms1p interaction [44], although the degree of any disruption in the interaction is unclear.

We will refer to the MLH1 and PMS1 alleles described above as the ‘mutator’ or ‘incompatible’ allele combination, though we show in this study that these alleles do not always cause a strong mutator phenotype when found together. Until recently, no natural isolates had been found carrying both alleles, in line with theoretical expectations of the absence of mutators in sexual populations. However, strains recently sequenced by Strope et al. [34] include a cluster of clinical isolates that carry both mutator alleles. This cluster of strains could represent a rare case of a facultatively sexual microbe with a naturally occurring mutator phenotype, and one that may be associated with invasion into a new ecological niche (the human body).

Here we report direct estimates of genomic mutation rates in the clinical background containing the two incompatible alleles. Furthermore, we investigate the phylogenetic history of the mutator isolates and examine levels of genomic variation in both mutator and non-mutator genetic backgrounds. We find that these isolates have a mildly elevated mutation rate (approximately four times greater than closely related non-mutator strains), significantly lower than previously reported for incompatible combinations of MLH1 and PMS1 alleles assayed in a single laboratory genetic background [42]. Genomic analysis of mutator and non-mutator strains provided no evidence of a historically elevated mutation rate. We conclude that the mutational effect of the incompatible mismatch repair alleles is dampened by background genetic modifiers, and discuss possible explanations for our observations.

## 2. Material and methods

### (a) Strains and media

Fifteen strains were chosen from the 100-genomes yeast panel [34]: four that contained both of the alleles necessary to confer a mutator phenotype (MLH1<sup>G761D</sup> and PMS1<sup>K818</sup>), six that contained only the PMS1<sup>K818</sup> allele and five that contained only the MLH1<sup>G761D</sup> allele. Two MLH1<sup>G761D</sup>–PMS1<sup>K818</sup> strains that were reported to have a mutation rate approximately 100× wild-type [41], EAY1370 and EAY1363 (S288c and SK1 genetic backgrounds, respectively; generously provided by Eric Alani), were used as a control. All strains assayed were heterothallic haploid MATa (i.e. derived from the original diploid background), as the mutator allele and the reporter for the mutation rate assay are recessive. In order for the control strains to be used in the fluctuation assays, the *URA3* gene was restored via lithium acetate transformation [45] using strain YJM128 as the PCR template. The URA3 protein sequence in all strains used in this study was identical. Strains were Sanger sequenced at the *PMS1* and *MLH1* loci with the following primers to verify allele status: MLH1for-CCAGGTTGATCATATAATGCC; MLH1rev-GGCGCTACACTTTCAATGAC; PMS1for-CATATAACATAGTTCCG; PMS1rev-CTCTGCAA ATGAGCTCAATCA. Strains are listed in table 1.

Strains were grown in YPD [46]; spontaneous *ura*-mutants were selected on synthetic complete agar plates [46] supplemented with 1 mg ml<sup>−1</sup> 5-fluoro-orotic acid (5FOA) and 60 mg ml<sup>−1</sup> uracil. Cultures were also treated with deflocculation buffer (20 mM sodium citrate, 5 mM EDTA), as strains YJM1639, YJM1851, YJM1747 and YJM1707 flocculated.

### (b) Fluctuation assays

The modified Jones protocol was used to estimate mutation rates [47]; following the procedure of Raynes et al. [26], spontaneous *ura*-mutants were assayed. All strains were grown overnight in YPD from freezer stocks; a new tube of 10 ml of YPD was inoculated with approximately 1000 cells and grown for 48 h. Five replicate cultures of 30 ml YPD were then inoculated with approximately 100–500 cells and grown for 48 h. The cultures were centrifuged, resuspended in 5 ml deflocculation buffer, washed and resuspended in 5 ml or 10 ml of water. For each replicate, 100 ul of an appropriate dilution was plated on YPD to estimate the population size and 300 ul was plated on 5FOA plates to select for spontaneous *ura*-mutants; for control strains, 10 ul + 90 ul water was also plated on 5FOA plates. Mutation rates were estimated using *Mutation Rate Calculator* software (provided by P. D. Sniegowski and P. J. Gerrish). This assay was replicated four times, with at least one control mutator strain included in two of the replicates.

### (c) Statistical tests for mutation rate variation

R v. 3.2.2 [48] and the lme4 package [49] were used to implement mixed models to test for differences in mutation rate. All models used the logarithm of mutation rate as the response variable. Assay was modelled as a random effect. Likelihood ratio tests were used to test for an assay effect in models containing a random strain effect and fixed effect for mutator alleles. To test for differences between groups of strains (engineered mutators, natural mutators and non-mutators), a random strain effect and a fixed effect for group differences were included. Coefficient estimates from these linear models quantified the fold differences between groups.

### (d) Haplotype analyses

For each of the 100 yeast genomes studied by Strope et al. [34] (except strain M22, which has a large amount of incomplete
sequence), at least 10 kb of DNA sequence was extracted surrounding the mutator alleles, amino acid 761 of MLH1 and 818 of PMS1. We used a method developed by Gabriel et al. [50] and implemented in the program HAPLOVIEW [51] to partition the sequences into blocks that did not show strong evidence of historical recombination. The sequence block containing the site of the mutator allele substitution in each gene was examined, and haplotype networks were constructed using POPART (http://popart.otago.ac.nz) with the median joining network option [52].

(e) DNA sequence variation analyses

Complete genomes of the 15 strains whose mutation rates were measured were obtained from Strope et al. [34]. The genome of S. paradoxus was obtained from Scannell et al. [53]. These 16 complete genomes were aligned with MUSSY v. 1r2.2 [54] using default options. MAF files were manipulated using MAF-TOOLS v. 0.1 [55]. To clarify comparisons, only alignment blocks where the majority of sequence fell into this subset of the full alignment—for the 12.16 Mb S288c genome, 11.75 Mb (97%) was present in complete alignment and 11.24 Mb (92%) was present in alignment blocks containing sequence from all 16 taxa. In order to obtain a reliable set of polymorphic sites, the following criteria were used to filter further: (i) ends of alignment blocks were trimmed if the sequence of any species consisted of only gaps; (ii) only biallelic single nucleotide variants were considered, not insertions–deletions (indels) or multi-allelic sites; and (iii) for a particular site, any strains with variants were considered, not insertions–deletions (indels) or multi-allelic sites; and (iii) for a particular site, any strains with

3. Results and discussion

(a) Four strains derived from natural isolates carry incompatible mismatch repair alleles

Strope et al. [34] sequenced the genomes of 93 S. cerevisiae strains derived from isolates collected globally with a particular focus on clinical isolates. These strains were haploid or homozygous diploid segregants of the isolates themselves. Surprisingly, four of the sequenced strains carried incompatible alleles in the mismatch repair genes MLH1 (G761D) and PMS1 (R818 K) that are thought to confer a dramatic increase in mutation rate when they co-occur [41]. While both alleles are known to be segregating in natural populations (24% and 12%, for MLH1D761 and PMS1K818, respectively), it was previously postulated that the alleles would not be found together in nature because the combination would be selected against due to the accumulation of deleterious mutations [41]. Indeed, a previous investigation into a diverse panel of yeast isolates uncovered no strains with the combination [42]. While this manuscript was in review, Bui et al. [56] published an analysis of MLH1 and PMS1 across an even larger panel of 1010 natural isolates and found only 19 isolates carrying the alleles together in either heterozygous or homozygous form, confirming that it is unusual to find this ‘mutator’ allele combination. The four strains recently discovered by Strope et al. [34] to be carrying the mutator allele combination are all clinical isolates classified as admixed ‘mosaics’, which do not show pure ancestry from a single S. cerevisiae population [34]. The isolates from which these strains were collected from geographically disparate locations in California (n = 3) and North Carolina (n = 1).

To understand the history of mutator alleles at MLH1 and PMS1, we analysed the haplotype structure of regions surrounding these loci in the 100-genomes yeast panel [34]. We partitioned this sequence into blocks showing little evidence of historical recombination, and found that each derived allele appears to have arisen once (on a single haplotype; figure 1) and spread widely within S. cerevisiae, as evidenced by its presence among diverse wine/European and mosaic strains. This observation is expected due to the small or non-existent fitness effect of each mutator allele when found with a wild-type allele at the opposing locus [41].
ently and significantly higher mutation rate estimates than demonstrated to exhibit a high mutation rate, had consistent and significantly higher mutation rate estimates than any other strains (figure 2; 40 times greater than the average non-engineered strains; $\chi^2 = 18.7, p = 1.6 \times 10^{-5}$). Surprisingly, the four natural mutator strains had only slightly higher mutation rates than the non-mutator strains (figure 2; 5.6 times greater than non-mutator strains; $\chi^2 = 29.5, p = 5.7 \times 10^{-8}$). Comparing the mutation rate estimates for these four strains to only their two closest non-mutator relatives among the strains we assayed (YJM1775 and YJM1787), the difference was even more subtle (4.3 times greater; $\chi^2 = 11.2, p = 8.2 \times 10^{-4}$). The full gene sequences from the natural mutator strain YJM1785 (isogenic to YJM555), when engineered into a laboratory strain background (S288c), led to a significantly larger increase in mutation rate (approximately 200-fold) [56].

(c) Patterns of genetic variation are similar in mutator and non-mutator strains

Although the mutator allele combination did not appear to give rise to substantially higher mutation rates in the natural mutator strains, this may not reflect the historical influence of this pair of alleles. In particular, an initial elevation of genomic mutation rate when the mutator alleles were first combined on a single genetic background could be dampened by the subsequent appearance of a modifier of mutation rate [25]. This scenario could manifest as an increase in genome-wide mutations in only those strains carrying the mutator combination (due to the historically higher mutation rate in those backgrounds) despite the present similar overall genomic mutation rates for both mutators and non-mutators.

To search for evidence of this pattern, we examined the number of derived mutations in each strain. We first aligned full genomes of the assayed strains to the genome of S. paradoxus, the closest extant relative of S. cerevisiae. Next, we examined sites that were polymorphic in S. cerevisiae
rates of engineered mutator strains from [41] to 100 genomes of natural mutator strains. Note the difference in mutation rate by strain. Shapes (see legend) show measurements from individual assays. Boxes show 25%, 50% and 75% quantiles. In both panels, strain labels indicate either diploid parent name (to facilitate comparison to genomes sequenced in [34]) or genetic background (for engineered mutator strains in [41]).

**Figure 2.** Mutation rates of strains carrying mutator and non-mutator allele combinations. (a) For each fluctuation assay, clinical strains with only one of the mutator alleles were averaged to provide the baseline mutation rate; the mutation rate for each strain was divided by this baseline. Shapes (see legend) show data from individual assays and diamonds indicate average for the strain over all assays. (i) Strains sequenced in [34] and assayed in this study. (ii) Comparison of mutation rates of engineered mutator strains from [41] to 100 genomes of natural mutator strains. Note the difference in y-axis scale between the panels. (b) Estimates of mutation rate by strain. Shapes (see legend) show measurements from individual assays. Boxes show 25%, 50% and 75% quantiles. In both panels, strain labels indicate either diploid parent name (to facilitate comparison to genomes sequenced in [34]) or genetic background (for engineered mutator strains in [41]). See table 1, for more details on each strain. (Online version in colour.)

and for which we could determine the derived allele using the *S. paradoxus* sequence. We found that the number of derived alleles in natural mutator strains was not higher than the number in non-mutator strains (natural mutator mean 44 892; non-mutator mean 45 793). The same pattern held when we restricted our focus to the natural mutator strains and their two closest relatives at the whole-genome level (YJM1775 and YJM1787) in order to focus only on mutations that occurred along branches separating the natural mutators from their close relatives (natural mutator mean 30 218; non-mutator mean 30 427). Nevertheless, it is difficult to falsify the hypothesis that background modifiers of mutation rate arose after the incompatible mutator alleles were brought together in a single genetic background, for at least two reasons. First, the mutation rate modifier allele(s) could have arisen shortly after the mutator alleles were brought together, resulting in a very small excess of mutations due to a short period of elevated mutation rate. Second, the complex population history of diverse yeast isolates, along with an unknown natural life cycle (i.e. clonal growth versus sexual reproduction), could obscure historical patterns of sequence divergence within and among lineages. We have shown that the mutation rate in natural mutator strains is slightly elevated (approximately \(4 \times\)) compared with their closest non-mutator relatives. Why, then, do we not observe any enrichment of derived mutations in natural mutator strains? There are two possibilities. First, we have fewer strains to compare that are closely related to the natural mutator strains. A larger number of evolutionary lineages could provide power to detect subtle trends in patterns of mutation over time. Second, the mutator alleles may have been present in heterozygous form, and thus not exhibiting the mutator phenotype, for a significant portion of their evolutionary history.

(d) No candidates for compensatory mutations modifying mutation rate

The lack of a strong effect of the mutator alleles on mutation rate in the clinical isolate backgrounds that we tested suggests a role for epistatic interactions with other loci. Previous work investigating this incompatibility utilized natural mutator alleles that contained additional single nucleotide polymorphism (SNP) variants within MLHI and PMSI [42]. These results demonstrated that intragenic modifiers could modulate the mutation rate over a 20-fold range, although in all cases the mutation rate was still significantly elevated (at least \(24 \times\) [42]). By contrast, our results suggest the presence of modifiers that greatly suppress the elevated mutation rate phenotype. Indeed, the slight elevation in mutation rate that we observed among natural mutator strains matches roughly with variation in mutation rate among different backgrounds with compatible allele combinations (up to sixfold increase relative to S288c) [42].

We used sequence data [34] to search for possible intragenic modifiers in the set of strains we assayed. Demogines *et al.* [42] found two alleles, PMSI\(^{F165C}\) and MLHI\(^{L271P}\), that modified mutation rate in strains with the incompatible mutator allele combination. The PMSI\(^{F165C}\) allele that increases mutation rate was not found among the strains we examined. However, the MLHI\(^{L271P}\) allele that decreases mutation rate threefold to fourfold was polymorphic among
the natural mutator strains. Strain YJM1785 (isogenic to YJM555) carries the reference T allele at position 595 697 on chromosome XIII, while the other three natural mutators carry the C allele that dampens the increased mutation rate due to the incompatible alleles. We observed a modestly lower mutation rate (approximately twofold) among the natural mutators carrying this ‘protected’ allele, in line with previous observations [42]. In addition to these variants, there were 18 SNP and indel variants in MLH1 and 40 in PMS1 that were polymorphic in the full panel of 100 Genomes strains we studied, of which 11 and 13 result in non-synonymous substitutions, respectively. Of these, only two SNPs in MLH1 and one SNP in PMS1 were polymorphic among the natural mutator strains. However, all had the same allelic pattern as the MLH1D761 and PMS1K818 mutator alleles (YJM1785 carried one allele and the other natural mutators carried the other), preventing us from determining whether any of these additional non-synonymous polymorphisms also modulate mutation rate in the incompatible background.

We expanded our focus to the full genome to search for candidate loci that could contain mutation-rate-lowering modifiers of the incompatible allele combination. First, we focused on a cluster of mutator and non-mutator strains (YJM1775, YJM1751 and YJM1753— isogenic to YJM320, YJM541 and YJM554, respectively) that are among the most closely related among 100 genomes strains. We compared YJM1775 along with S288c and Sigma1278b (non-mutators that may not contain compensatory mutations) versus YJM1751 and YJM1753 (natural mutators that presumably do contain compensatory mutations). However, there were no polymorphic sites that had one allele shared by the mutators and another by the non-mutators. Next, we expanded our focus to search for sites where all natural mutators shared one allele but their two closest non-mutator relatives at the whole-genome level (YJM1775 and YJM1787—isogenic to YJM320 and YJM681, respectively) shared another allele. Unfortunately, we found over 1000 sites that fit these criteria. We used the STRING database [57] to filter these sites and found only one potential polymorphism of interest, in the MutS homologue MSH5. However, this polymorphism is synonymous, and a predicted interaction between MLH1 and MSH5 is probably due to their shared role in promoting crossing over [58,59], rather than DNA mismatch repair. Overall, our inability to detect candidates may be because these strains are sufficiently divergent that a few large-effect compensatory mutations are obscured by the large amount of variation at other loci, or because there are many small-effect modifiers of mutation rate scattered throughout the genome. Moreover, these modifiers may have already been present in the genomic background on which the mutator alleles were first brought together. The complex machinery that is required to replicate DNA with high fidelity [60] makes it possible for many loci to influence the mutation rate. Given the challenges of making precise measurements of the mutation rate phenotype, uncovering modifiers of small effect using QTL approaches is not feasible.

4. Conclusion
In this study, we characterized the mutation rate in strains derived from natural isolates carrying a pair of incompatible alleles in mismatch repair genes that were previously shown to lead to a much higher genomic mutation rate when combined in one background [41]. The existence of naturally derived strains carrying this pair of alleles is unexpected given the prediction, from previous theoretical and experimental work, that natural populations of sexual microbes should not contain considerable frequencies of individuals carrying mutator alleles. We show that the mutation rate of laboratory strains engineered to carry the incompatible MLH1D761 and PMS1K818 mutator alleles is high, as demonstrated previously [41], but that these alleles only mildly elevate mutation rate in strains derived from natural clinical isolates. Thus, the effect of the incompatible alleles on mutation rate is modulated by genomic background.

The observation that all natural mutator strains identified by Strope et al. [34] are derived from clinical isolates raises the possibility that the human body, an unusual environment for \textit{S. cerevisiae}, poses unique selective pressures that could select for the mutator combination hitchhiking along with advantageous alleles during a period of clonal growth. Our data do not strongly support this contention given the relatively slight elevation of mutation rate in natural mutator strains. However, the haploid natural mutator strains we tested were derived from diploid clinical isolates that were probably highly heterozygous [40]. Thus, it remains a possibility, albeit unlikely, that within the population of \textit{S. cerevisiae} present in a single patient, these alleles could be present at moderate frequencies along with other mutation rate modifiers such that rare offspring containing the mutator alleles and no suppressors could contribute adaptive mutations that are quickly detached from their high-mutation-rate background via recombination.

Given the possible link between mutators and opportunistic pathogens, the question of the effect of mutator alleles in pathogenic strains of a facultatively sexual microbe is of particular interest. Our results demonstrate that a cluster of clinical yeast strains with putative mutator alleles are in fact not mutators. More broadly, it is intriguing that hundreds of isolates of the facultatively sexual yeast \textit{S. cerevisiae}—from around the globe and from various ecological niches— have been sequenced, and the only isolates found to contain the incompatible mutator combination do not actually have a strongly elevated mutation rate. It is worth noting that mutator alleles are more likely to be maintained in facultatively sexual organisms than in obligately sexual organisms due to periods of clonal growth where such alleles cannot be decoupled from beneficial mutations. Overall, our results provide rare and compelling evidence that supports evolutionary theory suggesting that sexual organisms are unlikely to harbour alleles that increase their genomic mutation rate.

Data accessibility. Mutation rate data, genome sequence data and code to reproduce statistical and genomic analyses is available at http://dx.doi.org/10.5061/dryad.125p3 [61].

Authors’ contributions. H.A.M. conceived of the study. B.M. and H.A.M. carried out assays to estimate mutation rate. D.A.S. and H.A.M. analysed the data. D.A.S. conducted bioinformatic analyses of genomic variation. H.A.M. and D.A.S. wrote the paper. P.M.M. provided strains and overall advice on the project. All authors gave final approval for publication.

Competing interests. The authors have no competing interests.

Funding. This work was supported by a College of William and Mary Faculty Research Grant (H.A.M.), NIH F32 GM110997 (D.A.S.) and NIH R01 GM098287 (P.M.M.).

Acknowledgements. We thank John McCusker and Eric Alani for strains, and P. D. Snigieowski for helpful comments on the manuscript.


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