

The Rate and Spectrum of Spontaneous Mutations in *Mycobacterium smegmatis*, a Bacterium Naturally Devoid of the Postreplicative Mismatch Repair Pathway

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ABSTRACT *Mycobacterium smegmatis* is a bacterium that is naturally devoid of known postreplicative DNA mismatch repair (MMR) homologs, *mutS* and *mutL*, providing an opportunity to investigate how the mutation rate and spectrum has evolved in the absence of a highly conserved primary repair pathway. Mutation accumulation experiments of *M. smegmatis* yielded a base-substitution mutation rate of 5.27×10^{-10} per site per generation, or 0.0036 per genome per generation, which is surprisingly similar to the mutation rate in MMR-functional unicellular organisms. Transitions were found more frequently than transversions, with the A:T→G:C transition rate significantly higher than the G:C→A:T transition rate, opposite to what is observed in most studied bacteria. We also found that the transition-mutation rate of *M. smegmatis* is significantly lower than that of other naturally MMR-devoid or MMR-knockout organisms. Two possible candidates that could be responsible for maintaining high DNA fidelity in this MMR-deficient organism are the ancestral-like DNA polymerase DnaE1, which contains a highly efficient DNA proofreading histidinol phosphatase (PHP) domain, and/or the existence of a uracil-DNA glycosylase B (UdgB) homolog that might protect the GC-rich *M. smegmatis* genome against DNA damage arising from oxidation or deamination. Our results suggest that *M. smegmatis* has a noncanonical Dam (DNA adenine methylase) methylation system, with target motifs differing from those previously reported. The mutation features of *M. smegmatis* provide further evidence that genomes harbor alternative routes for improving replication fidelity, even in the absence of major repair pathways.

KEYWORDS

mutation
accumulation
GC bias
Mycobacteria

Spontaneous mutations play a central role in most evolutionary processes, and are responsible for nearly all forms of genetic disease. For this reason, it is important that we understand how the mutation rate and

spectrum evolves across a wide range of organisms. Mutations arise from complex interactions between processes that damage DNA (exogenous and endogenous), prevent damage, and repair damage (Zhou and Elledge 2000), and, like most traits, the rate of mutation is determined by an interaction of the environment and these genetic factors. Previously, the mutation rate and spectrum has been studied by comparing putatively neutral sites in specific genes (Graur and Li 2000; Wielgoss *et al.* 2011), or by fluctuation tests using reporter-construct genes (Drake 1991). However, neither of these methods is free of potentially significant biases, because selection is likely to affect many putatively neutral sites and different genomic regions can have significantly different mutation rates (Hawk *et al.* 2005; Lynch 2007). By applying high-throughput sequencing technology to mutation-accumulation (MA) experiments, it is possible to generate an unbiased direct estimate of the genome-wide rate and

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spectrum of spontaneous mutations in an organism (Lynch *et al.* 2008; Halligan and Keightley 2009), allowing us to examine the forces driving the mutation process.

The general strategy of a bacterial MA experiment is to repeatedly bottleneck parallel lineages originated from a single cell for hundreds to thousands of generations. In this process, the strong bottlenecks minimize the efficacy of selection, enabling all but the most severely deleterious mutations to accumulate in an effectively neutral fashion (Muller 1927, 1928; Bateman 1959; Mukai 1964; Kibota and Lynch 1996). Through the MA process, unbiased estimates of genome-wide spontaneous mutation rates and spectra have been characterized for a number of eukaryotic and prokaryotic organisms (Lynch *et al.* 2008; Denver *et al.* 2009; Keightley *et al.* 2009, 2014, 2015; Ossowski *et al.* 2010; Lee *et al.* 2012; Sung *et al.* 2012a, 2012b; Behringer and Hall 2015; Dillon *et al.* 2015; Farlow *et al.* 2015; Long *et al.* 2015b; Ness *et al.* 2015), and have led to a general hypothesis explaining how mutation rates have evolved (Lynch 2010, 2011; Sung *et al.* 2012a). However, it remains unclear how DNA replication and repair interact to ultimately determine the mutation rate. Thus, further comparative work is needed to understand alternative evolutionary solutions to setting cellular mutation rates.

The *Mycobacterium* genus consists of a biologically diverse group of bacteria, 120 or more described species, including both human obligate pathogens, such as *M. tuberculosis* and *M. leprae*, and free-living saprophytes, such as *M. smegmatis* (Smith *et al.* 2009). *M. smegmatis* is relatively fast-growing, nonpathogenic, and genetically facile, so it provides an accessible model to study *Mycobacteria* in general (Snapper *et al.* 1990; Shiloh and DiGiuseppe Champion 2010). *M. smegmatis* has a genome of ~7 Mb (Mohan *et al.* 2015), which is larger than most other *Mycobacterium* strains, including members of the pathogenic *M. tuberculosis* complex (MTB complex, ~4.4 Mb), and *M. leprae* (~3.3 Mb) (Brosch *et al.* 2001). The reduction in genome size of these mycobacterial pathogens is attributed to pathogenicity evolution (Brosch *et al.* 2001). *Mycobacteria* are classified as *Actinomycetales*, and some members of this group, such as *Nocardia* and *Corynebacterium*, have unusually high genomic GC-contents when compared to other bacteria (65.6%-GC in *M. smegmatis*). Genome sequencing has revealed that *Mycobacteria*, like all *Actinomycetales*, do not have any identifiable genes encoding the widely conserved mutLS-based postreplicative mismatch repair (MMR) system (Cole *et al.* 1998; Ford *et al.* 2013), suggesting that *Mycobacteria* lack canonical MMR, and thus might have unusual mutation features.

MMR maintains the fidelity of genomes by typically removing a fraction of the replication errors (Kunkel and Erie 2005; Lee *et al.* 2012). Previous studies showed that mutation rates of some MMR-knockout organisms are 10–100 × higher than in MMR-functional organisms (Lee *et al.* 2012; Lang *et al.* 2013). In addition, given the fact that MMR deficiency is common in some species (Garcia-Gonzales *et al.* 2012), an organism can significantly reduce DNA damage by using other repair or prevention pathways. Thus, because *M. smegmatis* is naturally devoid of known MMR genes, it may have compensatory mechanisms for efficient protection against mutations.

MATERIALS AND METHODS

Mutation accumulation

Eighty independent *M. smegmatis* MC2 155 (ATCC 700084) MA lines were initiated from a single colony. 7H10 agar medium, with 0.5% glycerol and OADC enrichment 10% as recommended by ATCC, was used for the mutation-accumulation line transfers.

Every 2 d, a single isolated colony from each MA line was transferred by streaking to a new plate, ensuring that each line regularly passed through a single-cell bottleneck (Kibota and Lynch 1996). Each line passed through ~4900 cell divisions (Supplemental Material, Table S1). The bottlenecking procedure used for this experiment ensures that mutations accumulate in an effectively neutral fashion. MA lines were incubated at 37° under aerobic conditions. Frozen stocks of all lineages were prepared by growing a final colony per isolate in 1 ml 7H9 broth medium with 0.2% glycerol and ADC enrichment 10%, incubated overnight at 37°, and frozen in 20% glycerol at –80°.

DNA extraction and sequencing

The 75 lines that survived through the end of MA were prepared for whole genome sequencing. DNA was extracted with the Wizard Genomic DNA Purification kit (Promega, Madison, WI). DNA libraries for Illumina HiSeq2500 sequencing (insert size 300 bp) were constructed using the Nextera DNA Sample Preparation kit (Illumina, San Diego, CA). Paired-end 150-nt read sequencing of MA lines was done by the Hubbard Center for Genome Studies, University of New Hampshire, with an average sequencing depth of 126 × across all lines (Table S1).

Mutation identification and analyses

A consensus approach for identifying fixed base substitutions and small-indels in the MA lines was modified from Sung *et al.* (2015). Briefly, paired-end reads from each MA line were mapped to the reference genome (GenBank accession number: NC_018289.1) using BWA 0.6.2 (Li and Durbin 2009), and read alignment and duplicate-read removal around indels was performed using GATK (McKenna *et al.* 2010; DePristo 2011). The output was parsed with SAMTOOLS (Li *et al.* 2009); mapped reads needed to pass filters for sequencing/PCR/mismapping errors; 26 lines were removed from the final analysis due to library construction failure or cross-line contamination (Table S1). Candidate mutations were called if they differed from the consensus sequence of all MA lines. Using the BAM and SAM formatted files from the BWA pipeline, BreakDancer 1.1.2 (Chen *et al.* 2009) and Pindel 0.2.4w (Ye *et al.* 2009) were also used to realign reads and identify small-indels. Both the consensus pipelines and the realignment programs support the final reported indels.

Statistics and calculations

We used R v3.1.0 (R Development Core Team 2014) for all statistical tests and calculations; 95% Poisson confidence intervals were calculated using a χ^2 estimation (Johnson and Kemp 1993).

Data availability

Raw sequence reported in this study has been deposited in NCBI SRA (Bioproject No.: PRJNA320082; Study No.: SRP074205).

RESULTS

To estimate the mutation rate in *M. smegmatis* MC2 155, a mutation-accumulation experiment was carried out for 381 d (~4900 generations) with 80 independent lineages, all derived from the same ancestral colony of *M. smegmatis*. Every 2 d, a single colony from each line was restreaked onto a fresh plate, minimizing the effective population size. We analyzed the mutation rate and spectrum across 49 MA lines that were successfully sequenced and not contaminated by other MA lines.

■ **Table 1 Mutation rates of MMR deficient bacteria (numbers are in 10^{-10} site per generation)**

Organism	Transitions		Transversions				Overall Mutation Rate	Overall Mutation Rate of Wild-Type MA Lines	Reference
	A:T G:C	G:C A:T	A:T T:A	G:C T:A	A:T C:G	G:C C:G			
<i>B. subtilis</i> (<i>mutS</i> ⁻)	280.17	375.54	6.86	2.30	4.76	4.02	331.00	3.28	Sung <i>et al.</i> (2015)
<i>D. radiodurans</i> (<i>mutL</i> ⁻)	18.70	17.20	0.89	0	0.89	0.44	18.60	4.99	Long <i>et al.</i> (2015a)
<i>E. coli</i> (<i>mutL</i> ⁻)	389.09	152.43	4.77	3.41	3.41	1.02	275.00	2.66	Lee <i>et al.</i> (2012)
<i>M. florum</i>	13.20	165.94	4.06	93.30	3.05	47.30	98.00	—	Sung <i>et al.</i> (2012a)
<i>M. smegmatis</i>	3.95	2.76	0.27	1.58	2.10	0.43	5.27	—	This study
<i>Pseudomonas fluorescens</i> (<i>mutS</i> ⁻)	284.45	191.00	1.82	5.12	1.72	2.18	234.00	- ^a	Long <i>et al.</i> (2015b)

^aNo whole-genome sequence data available for wild-type strain.

Mutation rates

Across the 49 sequenced *M. smegmatis* MA lines (with an average of 6.77 Mb analyzable sequence per line, 97% of the total genome), we identified 856 base-substitution changes (Table S1 and Table S2), yielding an overall base-substitution mutation rate of 5.27×10^{-10} (SE = 1.93×10^{-11}) per site per generation, or 0.0036 per genome per generation. Our analysis also reveals 207 short insertions and deletions 1–27 bps in length (141 insertions, and 66 deletions), yielding an insertion/deletion rate of 1.27×10^{-10} (SE = 1.08×10^{-11}) per site per generation (Table S1 and Table S3). Although the insertion rate is $2.1 \times$ greater than the deletion rate, the total size of all insertions is 206 bp while the deletions total 225 bp, resulting in a net loss of 19 bp in DNA sequence across all lines, consistent with the universal prokaryotic deletion bias hypothesis (Mira *et al.* 2001). Of the small indels, 78.74% occur in simple sequence repeats (SSRs), *e.g.*, homopolymer runs (Table S3), and these small-indels comprised 15.33% of all mutations.

Using the annotated *M. smegmatis* MC2 155 genome (NCBI accession: NC_018289.1), we identified the functional context of each base substitution (Table S2). Across the 49 lines, 716 of the 856 (83.64%) substitutions are in coding regions (90% of the genome represents coding regions), while the remaining 140 are found at noncoding sites (Table S2). To test for the absence of selection in our experiment, we asked whether the ratio of nonsynonymous to synonymous mutations is significantly different from the random expectation. Given the codon usage and the transition/transversion ratio (see below) in *M. smegmatis*, the expected ratio of nonsynonymous to synonymous mutations is 2.60, which is not significantly different from the observed ratio of 2.11 (486/230) ($\chi^2 = 3.00$, $P > 0.01$). Thus, selection does not appear to have had a significant influence on the distribution of mutations in this experiment.

Comparison of mutation rates with various bacteria

Mutation rates in MMR-deficient genome backgrounds in several prokaryotic and eukaryotic organisms have been investigated by using whole-genome sequencing of mutation-accumulation lines. Most of these studies have found that MMR deficiency results in a >100-fold increase in the mutation rate compared to wild-type lines. In striking contrast, MMR-devoid *M. smegmatis* has a mutation rate comparable to that of other naturally MMR-proficient wild-type organisms (Table 1). These results suggest that *M. smegmatis* employs mechanisms that somehow compensate for the absence of MMR in order to reach the same mutation rate as other organisms that harbor the essential MMR enzymes.

Previous studies have observed low levels of nucleotide diversity in *M. tuberculosis* and *M. leprae* populations (Sreevatsan *et al.* 1997; Monot *et al.* 2009), and have proposed that this is a result of recent

population bottlenecks (Smith *et al.* 2009). However, low levels of nucleotide diversity can also be explained by low mutation rates (Lynch 2010). Low mutation rates observed in pathogenic strains of *M. tuberculosis* (2- to 7-fold lower than that observed in *M. smegmatis* in this study) are consistent with the latter explanation (Ford *et al.* 2011). However, the mutation rate difference between *M. smegmatis* and *M. tuberculosis* could also result from different experimental systems. Ford *et al.* (2011) used living infected macaques during latent infections to accumulate mutations, and detected only 14 base-substitution mutations, but no A:T→T:A or A:T→C:G transversions. The *in vivo* environment could have biased the mutations by strong selection such as the host immune system, even if high numbers of mutations had been detected. Thus, it cannot be confirmed that *M. tuberculosis* has a similar mutation spectrum with *M. smegmatis* by comparing our data with *M. tuberculosis* mutations detected from whole-genome sequencing in Ford *et al.* (2011). But, a mutation accumulation experiment using *M. tuberculosis* may provide a clear answer to this.

Mutation spectrum

Across the 49 MA lines, we found 511 transitions and 345 transversions, resulting in a transition/transversion ratio of 1.48. Among the base-substitution changes, there are 302 G:C→A:T transitions and 173 G:C→T:A transversions at GC sites, yielding a mutation rate in the AT direction of $\mu_{G/C \rightarrow A/T} = 4.34 \times 10^{-10}$ per site per generation. In contrast, 209 A:T→G:C transitions and 111 A:T→C:G transversions yielded a mutation rate in the G:C direction of $\mu_{A/T \rightarrow G/C} = 6.04 \times 10^{-10}$ per site per generation (Table S1), which is significantly higher than the $\mu_{G/C \rightarrow A/T}$ rate (95% Poisson confidence intervals for $\mu_{G/C \rightarrow A/T}$ 3.96–4.74 $\times 10^{-10}$, for $\mu_{A/T \rightarrow G/C}$ 5.40–6.75 $\times 10^{-10}$). Given these conditional A/T ↔ G/C mutation rates, the expected GC content from mutation alone is 58.2% (SE = 4.67%), significantly lower than the actual chromosomal GC content of 65.6%.

Methylated bases are mutational hotspots in bacteria (Schaaper and Dunn 1991; Lee *et al.* 2012). Previous studies have found that mycobacterial species contain methyltransferases that are not canonical Dam or Dcm DNA methyltransferases (Shell *et al.* 2013; Sharma *et al.* 2015; Zhu *et al.* 2016), but are associated with the presence of 6-methyladenine in their genomes (Shell *et al.* 2013). We examined mutation rates at noncanonical Dam target sites (Schlagman and Hattman 1989; Clark *et al.* 2012; Shell *et al.* 2013), and previously suggested noncanonical methylation sites in other bacteria (Long *et al.* 2015a), and found that 45% of the A:T→C:G transversions (50 of 111) fall in motifs of 5'GACC3' (30) and 5'CAACC3' (20), a 6.8-fold elevation from the transversion rate of A:T sites not falling in these motifs. The mutation hotspots at noncanonical Dam target sites suggest methylation at these sites (Table S4). Surprisingly, the

reported Mycobacterial Adenine Methyltransferase sites 5'GAATTC3' (Nikolaskaya *et al.* 1985) and 5'CTGGAG3' (Shell *et al.* 2013) are not enriched for A:T→C:G transversions, suggesting that these sites are not routinely methylated in the *M. smegmatis* genome.

Although the presence of 5-methylcytosines was previously reported in *M. tuberculosis* and *M. smegmatis* genomes (Srivastava *et al.* 1981; Hemavathy and Nagaraja 1995), recent studies have found no 5-methylcytosine modification in the genomes of *M. tuberculosis* complex strains (Shell *et al.* 2013; Zhu *et al.* 2016). In our study, 21% (65 of 302) of the G:C→A:T transitions fall in the motifs of 5'CCGC3', 5'CGCC3', 5'CGCG3', and 5'CGGC3', which were not reported previously, and 48% (145 of 302) fall in 5'CpG3' sites, ~3.5-fold elevated from cytosines not in these sites (Table S5). As shown in yeasts, cytosines at 5'CpG3' sites may have an elevated mutation rate even without methylation (Zhu *et al.* 2014; Behringer and Hall 2015; Farlow *et al.* 2015).

DISCUSSION

Because most mutations have slightly deleterious fitness effects (Baer *et al.* 2007; Eyre-Walker and Keightley 2007), natural selection is thought to operate to minimize replication errors and maximize DNA repair efficiency (Kimura 2009). It has been proposed that the efficacy of selection in reducing mutation rates is determined by the power of random genetic drift, which is inversely proportional to the effective population size (Lynch 2010, Lynch 2011). Given this theoretical framework, because population sizes in free-living bacteria are expected to be large (on the order of 10^7 – 10^9), we expect different species to have roughly similar per genome mutation rates if they have similar population sizes (Sung *et al.* 2012a; Sniegowski and Raynes 2013). Consistent with this idea, *M. smegmatis* has roughly the same mutation rate as other free-living bacteria (Lee *et al.* 2012; Long *et al.* 2015b; Sung *et al.* 2015). Yet *M. smegmatis* lacks critical MMR enzymes, suggesting that either *Mycobacterium* pre-MMR replication fidelity is higher than that of other prokaryotes, or that alternative biochemical mechanisms are used to arrive at the equivalent mutation rates.

Alternative pathways for replication fidelity

Three main processes influence DNA-replication fidelity: nucleotide insertion fidelity of the DNA polymerase, removal of mispaired nucleotides by the DNA proofreading exonuclease, and MMR. Sequential action of these three steps is responsible for the typically low bacterial error rate of $\sim 10^{-10}$ per base replicated (Schaaper 1993; Kunkel 2004). However, it remains possible that a deficiency in any one of these processes may be compensated for by increased fidelity in the others (Lynch 2012): in *M. smegmatis*, as in the case of *Deinococcus radiodurans* (Long *et al.* 2015a), it appears that a mechanism arising from such evolutionary layering must compensate for MMR deficiency.

Replication of the *Escherichia coli* chromosome is performed by the DNA polymerase III holoenzyme, which replicates the leading and lagging strands simultaneously (Kelman and O'Donnell 1995). The alpha (α) and epsilon (ϵ) subunits have a major effect on fidelity of the DNA polymerase III holoenzyme, allowing DNA synthesis to proceed with $\sim 10^{-7}$ errors/bp replicated (prior to proofreading) (Schaaper 1993; Kunkel and Erie 2005). The proofreading subunit of the DNA polymerase, the epsilon (ϵ) exonuclease, is also essential for high-fidelity DNA replication in *E. coli*, with inactivation increasing the mutation rate up to 200-fold (Schaaper 1993). However, surprisingly, Rock *et al.* (2015) found that although the proofreading exonuclease in *M. tuberculosis* is present, it is completely dispensable for fidelity, and an alternative exonuclease contributes to replicative fidelity in *Mycobacteria*. They found that the *Mycobacterial* DNA

polymerase DnaE1 performs DNA proofreading with a polymerase and histidinol phosphatase (PHP) domain; inactivation of the PHP domain increased the mutation rate by more than 3000-fold (Rock *et al.* 2015). This decrease in proofreading fidelity suggests that the burden of DNA repair placed on MMR in other species may instead be placed onto the DnaE1 proofreader in *Mycobacteria*.

Role of DNA methylation in biased mutation spectrum

In the *M. smegmatis* genome a subset of adenine and cytosine sites have an elevated mutation rate. These sites are associated with specific sequence motifs: 45% of the A:T→C:G transversions (50 of 111) occur at adenines in the motifs 5'GACC3' (30) and 5'CAACC3' (20), which are known noncanonical Dam methylation sites. In addition, 21% (65 of 302) of the G:C→A:T transitions occur at cytosines in the motifs 5'CCGC3', 5'CGCC3', 5'CGCG3', and 5'CGGC3', and overall 48% (145 of 302) of the G:C→A:T transitions fall in 5'CpG3' sites. These two classes of mutation account for nearly a quarter of all base-substitution changes that we observed, and on balance they sum to a strongly biased G:C→A:T transition, making the overall A:T→C:G rate dependent on the mutation spectrum at unmethylated sites. While adenine methylation has been reported in *M. smegmatis*, cytosine methylation has not been seen (Shell *et al.* 2013; Zhu *et al.* 2016), although our results suggest this should be reexamined.

Alternative forms of DNA repair

Different DNA repair processes may generate the unusual mutation spectrum observed in *M. smegmatis*. A near universal mutation bias toward A/T has been observed in most species (Hershberg and Petrov 2010), but we find a bias to G/C in *M. smegmatis*. Notably, *M. smegmatis* is GC-rich, suggesting that mutation bias may have a role in determining the GC content in this genome. For the species in which a GC mutation bias observed (Dillon *et al.* 2015; Long *et al.* 2015a), this may be a product of methylation, deamination, and/or repair. For example, *Mycobacteria* have a high level of redundancy in the base excision repair (BER) pathway (van der Veen and Tang 2015), which could reduce the number of G:C→T:A transversions (Wallace 2002) associated with oxidative damage (David *et al.* 2007). In both *Bacillus subtilis* and *E. coli*, MutY can compensate for MMR enzymes, by removing adenines that are mispaired with cytosines, and preventing G:C→A:T mutations (Kim *et al.* 2003; Bai and Lu 2007; Debora *et al.* 2011).

GC-rich genomes may deploy additional enzymes to survey the fidelity of GC-sites, which are highly susceptible to cytosine deamination (Dos Vultos *et al.* 2009). The UdgB enzyme plays a more important role in removing uracils in *M. smegmatis* than for bacteria with known MMR activities (Wanner *et al.* 2009; Malshetty *et al.* 2010). For example, based on conserved sequences, six Udg families have been identified in various eubacteria, with different substrate specificities (Pearl 2000; Sartori *et al.* 2002; Srinath *et al.* 2007; Lee *et al.* 2011). *Mycobacteria* encode one family 1 Ung, and one family 5 UdgB (Sartori *et al.* 2002). The latter has only been characterized in a few organisms such as hyperthermophilic archaea and *M. tuberculosis*, and eukaryotes do not have this enzyme (Sartori *et al.* 2002; Starkuviene and Fritz 2002; Hoseki *et al.* 2003; Srinath *et al.* 2007). *In vitro* assays show that UdgB removes uracil from both ssDNA and dsDNA (Sartori *et al.* 2002), and excises hypoxanthine (Hx) from oligonucleotide substrates *in vitro* (Sartori *et al.* 2002; Srinath *et al.* 2007). Wanner *et al.* (2009) found that the mutation frequency in a *udg* knock-out strain of *M. smegmatis* is ~8-fold higher relative to wild type (Wanner *et al.* 2009). Furthermore, *M. smegmatis* Ung is more

efficient at excising uracils from hairpin-loop substrates than that of *E. coli* (Purnapatre and Varshney 1998), and the frequency of mutations in double *udgB ung* mutants is 56-fold higher than in wild-type *M. smegmatis* (Wanner *et al.* 2009). Similar to these results, Malshetty *et al.* (2010) showed synergistic effects of UdgB and Ung in mutation prevention in *M. smegmatis*: the mutation rate of a *udgB* knockout is ~2.1-fold higher, and the rate of a *ung* knockout is ~8.4-fold higher than wild-type *M. smegmatis*. But the double knockout (*udgB-ung*) shows a ~19.6-fold increase in mutation rate (Malshetty *et al.* 2010). By contrast, uracil DNA glycosylase (*ung*) mutants increase mutation frequency by only ~2-fold in *B. subtilis* (López-Olmos *et al.* 2012), and ~5-fold in *E. coli* (Duncan and Weiss 1982).

In conclusion, we have shown that *M. smegmatis* has a typical bacterial mutation rate, even though it lacks the near-universal MMR system, has an unusual A:T → C:G biased mutation spectrum, and has motifs for both probable adenine and cytosine methylation, which act as genomic mutational hotspots. We have discussed possible mechanisms that allow *M. smegmatis* to evolve a low mutation rate despite the apparent absence of MMR. Consistent with the drift-barrier hypothesis (Lynch 2010; Sung *et al.* 2012a), *M. smegmatis* has evolved to a summed replication fidelity and repair rate equal to that observed in most free-living bacteria. However, the lack of MMR in *M. smegmatis* necessitates compensatory selection to improve alternative enzymatic pathways that limit the mutation rate expected for its population size. Further biochemical assays are required to determine whether the discussed pathways replace the role of MMR with DNA replication fidelity, or if novel repair pathways exist.

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LITERATURE CITED

- Baer, C. F., M. M. Miyamoto, and D. R. Denver, 2007 Mutation rate variation in multicellular eukaryotes: causes and consequences. *Nat. Rev. Genet.* 8: 619–631.
- Bai, H., and A.-L. Lu, 2007 Physical and functional interactions between *Escherichia coli* MutY glycosylase and mismatch repair protein MutS. *J. Bacteriol.* 189: 902–910.
- Bateman, A. J., 1959 The viability of near-normal irradiated chromosomes. *Int. J. Radiat. Biol.* 1: 170–180.
- Behringer, M. G., and D. W. Hall, 2015 Genome wide estimates of mutation rates and spectrum in *Schizosaccharomyces pombe* indicate CpG sites are highly mutagenic despite the absence of DNA methylation. *G3 (Bethesda)* 6: 149–160.
- Brosch, R., A. S. Pym, S. V. Gordon, and S. T. Cole, 2001 The evolution of mycobacterial pathogenicity: clues from comparative genomics. *Trends Microbiol.* 9: 452–458.
- Chen, K., J. W. Wallis, M. D. McLellan, D. E. Larson, J. M. Kalicki *et al.*, 2009 BreakDancer: an algorithm for high-resolution mapping of genomic structural variation. *Nat. Methods* 6: 677–681.
- Clark, T. A., I. A. Murray, R. D. Morgan, A. O. Kislyuk, K. E. Spittle *et al.*, 2012 Characterization of DNA methyltransferase specificities using single-molecule, real-time DNA sequencing. *Nucleic Acids Res.* 40: e29.
- Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher *et al.*, 1998 Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* 393: 537–544.
- David, S. S., V. L. O’Shea, and S. Kundu, 2007 Base-excision repair of oxidative DNA damage. *Nature* 447: 941–950.
- Debora, B. N., L. E. Vidales, R. Ramirez, M. Ramirez, E. A. Robledo *et al.*, 2011 Mismatch repair modulation of MutY activity drives *Bacillus subtilis* stationary-phase mutagenesis. *J. Bacteriol.* 193: 236–245.
- Denver, D. R., P. C. Dolan, L. J. Wilhelm, W. Sung, J. I. Lucas-Lledó *et al.*, 2009 A genome-wide view of *Caenorhabditis elegans* base-substitution mutation processes. *Proc. Natl. Acad. Sci. USA* 106: 16310–16314.
- DePristo, M. A., E. Banks, R. Poplin, and K. V. GarimellaMaguire, J. R. *et al.*, 2011 A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nat. Genet.* 43: 491–498.
- Dillon, M. M., W. Sung, M. Lynch, and V. S. Cooper, 2015 The rate and molecular spectrum of spontaneous mutations in the GC-rich multi-chromosome genome of *Burkholderia cenocepacia*. *Genetics* 200: 935–946.
- Dos Vultos, T., O. Mestre, T. Tonjum, and B. Gicquel, 2009 DNA repair in *Mycobacterium tuberculosis* revisited. *FEMS Microbiol. Rev.* 33: 471–487.
- Drake, J. W., 1991 A constant rate of spontaneous mutation in DNA-based microbes. *Proc. Natl. Acad. Sci. USA* 88: 7160–7164.
- Duncan, B. K., and B. Weiss, 1982 Specific mutator effects of *ung* (uracil-DNA glycosylase) mutations in *Escherichia coli*. *J. Bacteriol.* 151: 750–755.
- Eyre-Walker, A., and P. D. Keightley, 2007 The distribution of fitness effects of new mutations. *Nat. Rev. Genet.* 8: 610–618.
- Farlow, A., H. Long, S. Arnoux, W. Sung, T. G. Doak *et al.*, 2015 The spontaneous mutation rate in the fission yeast *Schizosaccharomyces pombe*. *Genetics* 201: 737–744.
- Ford, C. B., P. L. Lin, M. R. Chase, R. R. Shah, O. Iartchouk *et al.*, 2011 Use of whole genome sequencing to estimate the mutation rate of *Mycobacterium tuberculosis* during latent infection. *Nat. Genet.* 43: 482–486.
- Ford, C. B., R. R. Shah, M. K. Maeda, S. Gagneux, M. B. Murray *et al.*, 2013 *Mycobacterium tuberculosis* mutation rate estimates from different lineages predict substantial differences in the emergence of drug-resistant tuberculosis. *Nat. Genet.* 45: 784–790.
- García-Gonzales, A., R. J. Rivera-Rivera, and S. E. Massey, 2012 The presence of the DNA repair genes mutM, mutY, mutL, and mutS is related to proteome size in bacterial genomes. *Front. Genet.* 3: 1–11.
- Graur, D., and W. H. Li, 2000 Fundamentals of Molecular Evolution, Sinauer Associates, Sunderland, MA.
- Halligan, D. L., and P. D. Keightley, 2009 Spontaneous mutation accumulation studies in evolutionary genetics. *Annu. Rev. Ecol. Evol. Syst.* 40: 151–172.
- Hawk, J. D., L. Stefanovic, J. C. Boyer, T. D. Petes, and R. A. Farber, 2005 Variation in efficiency of DNA mismatch repair at different sites in the yeast genome. *Proc. Natl. Acad. Sci. USA* 102: 8639–8643.
- Hemavathy, K. C., and V. Nagaraja, 1995 DNA methylation in mycobacteria: absence of methylation at GATC (Dam) and CCA/TGG (Dcm) sequences. *FEMS Immunol. Med. Microbiol.* 11: 291–296.
- Hershberg, R., and D. A. Petrov, 2010 Evidence that mutation is universally biased towards AT in bacteria. *PLoS Genet.* 6: e1001115.
- Hoseki, J., A. Okamoto, R. Masui, T. Shibata, Y. Inoue *et al.*, 2003 Crystal structure of a family 4 uracil-DNA glycosylase from *Thermus thermophilus* HB8. *J. Mol. Biol.* 333: 515–526.
- Johnson, N. L., and A. W. Kemp, 1993 Univariate Discrete Distributions, Wiley-Interscience, Hoboken, NJ.
- Keightley, P. D., U. Trivedi, M. Thomson, F. Oliver, S. Kumar *et al.*, 2009 Analysis of the genome sequences of three *Drosophila melanogaster* spontaneous mutation accumulation lines. *Genome Res.* 19: 1195–1201.
- Keightley, P. D., R. W. Ness, D. L. Halligan, and P. R. Haddrill, 2014 Estimation of the spontaneous mutation rate per nucleotide site in a *Drosophila melanogaster* full-sib family. *Genetics* 196: 313–320.
- Keightley, P. D., A. Pinharanda, R. W. Ness, F. Simpson, K. K. Dasmahapatra *et al.*, 2015 Estimation of the spontaneous mutation rate in *Heliconius melpomene*. *Mol. Biol. Evol.* 32: 239–243.
- Kelman, Z., and M. O’Donnell, 1995 DNA polymerase III holoenzyme: structure and function of a chromosomal replicating machine. *Annu. Rev. Biochem.* 64: 171–200.
- Kibota, T. T., and M. Lynch, 1996 Estimate of the genomic mutation rate deleterious to overall fitness in *E. coli*. *Nature* 381: 694–696.
- Kim, M., T. Huang, and J. H. Miller, 2003 Competition between MutY and mismatch repair at A-C mispairs in vivo. *J. Bacteriol.* 185: 4626–4629.

- Kimura, M., 2009 On the evolutionary adjustment of spontaneous mutation rates. *Genet. Res.* 9: 23.
- Kunkel, T. A., 2004 DNA replication fidelity. *J. Biol. Chem.* 279: 16895–16898.
- Kunkel, T. A., and D. A. Erie, 2005 DNA mismatch repair. *Annu. Rev. Biochem.* 74: 681–710.
- Lang, G. I., L. Parsons, and A. E. Gammie, 2013 Mutation rates, spectra, and genome-wide distribution of spontaneous mutations in mismatch repair deficient yeast. *G3 (Bethesda)* 3: 1453–1465.
- Lee, H., E. Popodi, H. Tang, and P. L. Foster, 2012 Rate and molecular spectrum of spontaneous mutations in the bacterium *Escherichia coli* as determined by whole-genome sequencing. *Proc. Natl. Acad. Sci. USA* 109: E2774–E2783.
- Lee, H. W., B. N. Dominy, and W. Cao, 2011 New family of deamination repair enzymes in uracil-DNA glycosylase superfamily. *J. Biol. Chem.* 286: 31282–31287.
- Li, H., and R. Durbin, 2009 Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* 25: 1754–1760.
- Li, H., B. Handsaker, A. Wysoker, T. Fennell, J. Ruan *et al.*, 2009 The sequence alignment/map (SAM) format and SAMtools. *Bioinformatics* 25: 2078–2079.
- Long, H., S. Kucukyildirim, W. Sung, E. Williams, H. Lee *et al.*, 2015a Background mutational features of the radiation-resistant bacterium *Deinococcus radiodurans*. *Mol. Biol. Evol.* 32: 2383–2392.
- Long, H., W. Sung, S. F. Miller, M. Ackerman, T. G. Doak *et al.*, 2015b Mutation rate, spectrum, topology, and context-dependency in the DNA mismatch repair (MMR) deficient *Pseudomonas fluorescens* ATCC948. *Genome Biol. Evol.* 7: 262–271.
- López-Olmos, K., M. P. Hernández, J. A. Contreras-Garduño, E. A. Robledo, P. Setlow *et al.*, 2012 Roles of endonuclease V, uracil-DNA glycosylase, and mismatch repair in *Bacillus subtilis* DNA base-deamination-induced mutagenesis. *J. Bacteriol.* 194: 243–252.
- Lynch, M., 2007 *The Origins of Genome Architecture*, Sinauer Associates, Sunderland, MA.
- Lynch, M., 2010 Evolution of the mutation rate. *Trends Genet.* 26: 345–352.
- Lynch, M., 2011 The lower bound to the evolution of mutation rates. *Genome Biol. Evol.* 3: 1107–1118.
- Lynch, M., 2012 Evolutionary layering and the limits to cellular perfection. *Proc. Natl. Acad. Sci. USA* 109: 18851–18856.
- Lynch, M., W. Sung, K. Morris, N. Coffey, C. R. Landry *et al.*, 2008 A genome-wide view of the spectrum of spontaneous mutations in yeast. *Proc. Natl. Acad. Sci. USA* 105: 9272–9277.
- Malshetty, V. S., R. Jain, T. Srinath, K. Kurthkoti, and U. Varshney, 2010 Synergistic effects of UdgB and Ung in mutation prevention and protection against commonly encountered DNA damaging agents in *Mycobacterium smegmatis*. *Microbiology* 156: 940–949.
- McKenna, A. H. M., E. Banks, A. Sivachenko, K. Cibulskis, A. Kernytsky *et al.*, 2010 The genome analysis toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Res.* 20: 1297–1303.
- Mira, A., H. Ochman, and N. A. Moran, 2001 Deletional bias and the evolution of bacterial genomes. *Trends Genet.* 17: 589–596.
- Mohan, A., J. Padiadpu, P. Baloni, and N. Chandra, 2015 Complete genome sequences of a *Mycobacterium smegmatis* laboratory strain (MC2 155) and isoniazid-resistant (4XR1/R2) mutant strains. *Genome Announc.* 3: e01520–e01514.
- Monot, M., N. Honore, T. Garnier, N. Zidane, D. Sherafi *et al.*, 2009 Comparative genomic and phylogeographic analysis of *Mycobacterium leprae*. *Nat. Genet.* 41: 1282–1289.
- Mukai, T., 1964 The genetic structure of natural populations of *Drosophila melanogaster*. I. Spontaneous mutation rate of polygenes controlling viability. *Genetics* 50: 1–19.
- Muller, H. J., 1927 Artificial transmutation of the gene. *Science* 66: 84–87.
- Muller, H. J., 1928 The measurement of gene mutation rate in *Drosophila*, its high variability, and its dependence upon temperature. *Genetics* 13: 279–357.
- Ness, R. W., A. D. Morgan, R. B. Vasanthakrishnan, N. Colegrave, and P. D. Keightley, 2015 Extensive de novo mutation rate variation between individuals and across the genome of *Chlamydomonas reinhardtii*. *Genome Res.* 25: 1739–1749.
- Nikolaskaya, I. I., N. G. Lopatina, E. V. Sharkova, and S. V. Suchkov, P. Somody *et al.*, 1985 Sequence specificity of isolated DNA-adenine methylases from *Mycobacterium smegmatis* (*butyricum*) and *Shigella sonnei* 47 cells. *Biochem. Int.* 10: 405–413.
- Ossowski, S., K. Schneeberger, J. I. Lucas-Lledó, N. Warthmann, R. M. Clark *et al.*, 2010 The rate and molecular spectrum of spontaneous mutations in *Arabidopsis thaliana*. *Science* 327: 92–94.
- Pearl, L. H., 2000 Structure and function in the uracil-DNA glycosylase superfamily. *Mutat. Res.* 460: 165–181.
- Purnapatre, K., and U. Varshney, 1998 Uracil DNA glycosylase from *Mycobacterium smegmatis* and its distinct biochemical properties. *Eur. J. Biochem.* 256: 580–588.
- R Development Core Team, 2014 R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing, Vienna, Austria.
- Rock, J. M., U. F. Lang, M. R. Chase, C. B. Ford, E. R. Gerrick *et al.*, 2015 DNA replication fidelity in *Mycobacterium tuberculosis* is mediated by an ancestral prokaryotic proofreader. *Nat. Genet.* 47: 677–681.
- Sartori, A. A., S. Fitz-Gibbon, H. Yang, J. H. Miller, and J. Jiricny, 2002 A novel uracil-DNA glycosylase with broad substrate specificity and an unusual active site. *EMBO J.* 21: 3182–3191.
- Schaaper, R. M., 1993 Base selection, proofreading, and mismatch repair during DNA replication in *Escherichia coli*. *J. Biol. Chem.* 268: 23762–23765.
- Schaaper, R. M., and R. L. Dunn, 1991 Spontaneous mutation in the *Escherichia coli* lac I gene. *Genetics* 129: 317–326.
- Schlagman, S. L., and S. Hattman, 1989 The bacteriophage T2 and T4 DNA-[N6-adenine] methyltransferase (Dam) sequence specificities are not identical. *Nucleic Acids Res.* 17: 9101–9112.
- Sharma, G., S. Upadhyay, M. Srilalitha, V. K. Nandicoori, and S. Khosla, 2015 The interaction of mycobacterial protein Rv2966c with host chromatin is mediated through non-CpG methylation and histone H3/H4 binding. *Nucleic Acids Res.* 43: 3922–3937.
- Shell, S. S., E. G. Prestwich, S. H. Baek, R. R. Shah, C. M. Sasseti *et al.*, 2013 DNA methylation impacts gene expression and ensures hypoxic survival of *Mycobacterium tuberculosis*. *PLoS Pathog.* 9: e1003419.
- Shiloh, M. U., and P. A. DiGiuseppe Champion, 2010 To catch a killer. What can mycobacterial models teach us about *Mycobacterium tuberculosis* pathogenesis? *Curr. Opin. Microbiol.* 13: 86–92.
- Smith, N. H., R. G. Hewinson, K. Kremer, R. Brosch, and S. V. Gordon, 2009 Myths and misconceptions: the origin and evolution of *Mycobacterium tuberculosis*. *Nat. Rev. Microbiol.* 7: 537–544.
- Snapper, S. B., R. E. Melton, S. Mustafa, T. Kieser, and W. R. Jacobs, Jr., 1990 Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis*. *Mol. Microbiol.* 4: 1911–1919.
- Sniegowski, P., and Y. Raynes, 2013 Mutation rates: how low can you go? *Curr. Biol.* 23: R147–R149.
- Sreevatsan, S., X. Pan, K. E. Stockbauer, N. D. Connell, B. N. Kreiswirth *et al.*, 1997 Restricted structural gene polymorphism in the *Mycobacterium tuberculosis* complex indicates evolutionarily recent global dissemination. *Proc. Natl. Acad. Sci. USA* 94: 9869–9874.
- Srinath, T., S. K. Bharti, and U. Varshney, 2007 Substrate specificities and functional characterization of a thermo-tolerant uracil DNA glycosylase (UdgB) from *Mycobacterium tuberculosis*. *DNA Repair (Amst.)* 6: 1517–1528.
- Srivastava, R., K. P. Gopinathan, and T. Ramakrishnan, 1981 Deoxyribonucleic acid methylation in mycobacteria. *J. Bacteriol.* 148: 716–719.
- Starkuviene, V., and H.-J. Fritz, 2002 A novel type of uracil-DNA glycosylase mediating repair of hydrolytic DNA damage in the extremely thermophilic eubacterium *Thermus thermophilus*. *Nucleic Acids Res.* 30: 2097–2102.
- Sung, W., M. S. Ackerman, S. F. Miller, T. G. Doak, and M. Lynch, 2012a Drift-barrier hypothesis and mutation rate evolution. *Proc. Natl. Acad. Sci. USA* 109: 18488–18492.

- Sung, W., A. E. Tucker, T. G. Doak, E. Choi, W. K. Thomas *et al.*, 2012b Extraordinary genome stability in the ciliate *Paramecium tetraurelia*. *Proc. Natl. Acad. Sci. USA* 109: 19339–19344.
- Sung, W., M. S. Ackerman, J.-F. Gout, S. F. Miller, E. Williams *et al.*, 2015 Asymmetric context-dependent mutation patterns revealed through mutation-accumulation experiments. *Mol. Biol. Evol.* 32: 1672–1683.
- van der Veen, S., and C. M. Tang, 2015 The BER necessities: the repair of DNA damage in human-adapted bacterial pathogens. *Nat. Rev. Microbiol.* 13: 83–94.
- Wallace, S. S., 2002 Biological consequences of free radical-damaged DNA bases. *Free Radic. Biol. Med.* 33: 1–14.
- Wanner, R. M., D. Castor, C. Güthlein, E. C. Böttger, B. Springer *et al.*, 2009 The uracil DNA glycosylase UdgB of *Mycobacterium smegmatis* protects the organism from the mutagenic effects of cytosine and adenine deamination. *J. Bacteriol.* 191: 6312–6319.
- Wielgoss, S., J. E. Barrick, O. Tenaillon, S. Cruveiller, B. Chane-Woon-Ming *et al.*, 2011 Mutation rate inferred from synonymous substitutions in a long-term evolution experiment with *Escherichia coli*. *G3 (Bethesda)* 1: 183–186.
- Ye, K., M. H. Schulz, Q. Long, R. Apweiler, and Z. Ning, 2009 Pindel: a pattern growth approach to detect break points of large deletions and medium sized insertions from paired-end short reads. *Bioinformatics* 25: 2865–2871.
- Zhou, B. B., and S. J. Elledge, 2000 The DNA damage response: putting checkpoints in perspective. *Nature* 408: 433–439.
- Zhu, L., J. Zhong, X. Jia, G. Liu, Y. Kang *et al.*, 2016 Precision methylome characterization of *Mycobacterium tuberculosis* complex (MTBC) using PacBio single-molecule real-time (SMRT) technology. *Nucleic Acids Res.* 44: 730–743.
- Zhu, Y. O., M. L. Siegal, D. W. Hall, and D. A. Petrov, 2014 Precise estimates of mutation rate and spectrum in yeast. *Proc. Natl. Acad. Sci. USA* 111: E2310–E2318.

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